

INTRACELLULAR HYDROGEN TRANSPORT SYSTEMS
IN HAEMOPOIETIC CELLS

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SUMMARY

A cytochemical study has been made of the dehydrogenase enzyme activity in human haemopoietic cells in relation to megaloblastic anaemia, chronic renal failure, and acute and chronic leukaemia. These studies were initiated as a result of reported abnormalities in the serum lactate dehydrogenase activity in these diseases, together with reports of dehydrogenase enzyme abnormalities in the cells of solid tumours.

1. Megaloblastic Anaemia

Previous biochemical studies of the serum lactate dehydrogenase activity in megaloblastic anaemia have shown elevated levels. It was postulated, on the basis of marrow homogenate studies, that the intramedullary destruction of megaloblasts might be the source of origin of this increased enzyme activity, but the conclusions were tentative in view of the inevitable mixed cell population in homogenate preparations. A cytochemical study has therefore been made of the activity of lactate dehydrogenase, and of other representative enzymes from the Embden-Meyerhof pathway, tricarboxylic acid cycle and pentose phosphate shunt, in megaloblastic anaemia and in normoblastic hyperplasia of equivalent degree.

This/

This necessitated the development of an improved technique for dehydrogenase enzyme cytochemistry comprising cell fixation with ethanol at -70°C and the incorporation in the reaction mixture of a polyvinyl alcohol as a cell preservative. The results show a progressive decrease in intra- and extra-mitochondrial enzyme activity in erythroid cells with maturation but with an increased activity in megaloblasts, compared with normoblasts, at equivalent stages of maturation. This increase, together with the known intramedullary destruction of megaloblasts, is therefore likely to contribute substantially to the elevated serum enzyme levels.

2. Chronic Renal Failure

Chronic renal failure, with high blood urea levels, is associated with a variable degree of reduction in marrow erythroid cell production and in red cell survival. A dialysable lactate dehydrogenase inhibitor has previously been described in the plasma in chronic renal failure. This suggested that the inhibitor, by reducing glycolytic ATP synthesis, might adversely affect glycolysis-dependent mitosis in marrow erythroblasts and, by inhibiting the glycolysis-dependent erythrocyte-membrane ionic pump, might also jeopardise red cell survival. A combined/

combined cytochemical and biochemical study of dehydrogenase enzyme activity in marrow erythroblasts and in blood erythrocytes was therefore carried out in patients with chronic renal failure with high blood urea levels. This failed to show any significant reduction in lactate dehydrogenase activity within erythroid cells and suggests that the plasma inhibitor is unlikely to contribute significantly to the anaemia of chronic renal failure.

3. Leukaemia

Warburg's (1930) hypothesis stated that cancer cells exhibit an obligatory aerobic glycolysis because they are unable to utilise the intra-mitochondrial respiratory electron pathway for energy production. Although no significant respiratory pathway defect has ever been demonstrated, there is general agreement that neoplastic cells do show a preferential aerobic glycolysis and the elevated serum lactate dehydrogenase levels in acute leukaemia may reflect this. Since it is difficult to assess the significance of glycolytic and respiratory enzyme activity in homogenate studies of solid tumours due to the mixed cell population (in respect of cell type, proliferative rate, stage of maturation and degree of hypoxia/

hypoxia), a cytochemical study has been made of the dehydrogenase enzyme activity in marrow and blood leucocytes in acute and chronic human leukaemia. For this purpose it was necessary to develop a cytochemical technique which did not require cell fixation and in which the reaction end-product was measured by scanning and integrating microdensitometry.

The activity of intra-mitochondrial respiratory enzymes, extra-mitochondrial glycolytic enzymes and mitochondrial membrane shuttle system enzymes has been related to stage of cellular maturation. The results show disproportionately high glycolytic enzyme activity in the lymphoblast with relatively low intra-mitochondrial and α -glycerolphosphate shuttle enzyme activity. A similar pattern was found in the Burkitt lymphoma cell and is probably related to the small number of mitochondria in immature lymphoid cells. This abnormality, in association with relatively low pentose phosphate shunt activity, results in a metabolic dependence on lactate dehydrogenase for continuing ATP synthesis and implies that specific lactate dehydrogenase inhibition might result in a selective cytotoxic effect for the lymphoblast.

An in vitro cell culture system was therefore devised to incorporate an 0.08M concentration of the specific lactate/

lactate dehydrogenase inhibitor sodium oxamate at physiological osmolality. This inhibitor, compared with 0.08M sodium chloride in control preparations, showed a significant cytotoxic effect for the lymphoblast and EB2 Burkitt lymphoma cell, but not for normal marrow myeloid precursors or blood neutrophils and lymphocytes. A reduction in glycolysis-dependent neutrophil phagocytosis was observed however. The results indicate the potential value of glycolytic enzyme inhibition as a means of chemotherapy in acute lymphoblastic leukaemia.

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ABBREVIATIONS USED IN THE TEXT

CO-ENZYMES

NAD	: nicotinamide adenine dinucleotide
NADH ₂	: nicotinamide adenine dinucleotide, reduced form
NADP	: nicotinamide adenine dinucleotide phosphate
NADPH ₂	: nicotinamide adenine dinucleotide phosphate, reduced form

ENZYMES (abbreviation : trivial name; Enzyme Commission number; systematic name)

1. Intra-mitochondrial

GD	: glutamate dehydrogenase ; 1.4.1.2 ; L-glutamate:NAD oxidoreductase
α -GPD	: glycerolphosphate dehydrogenase ; 1.1.99.5 ; L-glycerol-3-phosphate:(acceptor) oxidoreductase
SD	: succinate dehydrogenase ; 1.3.99.1 ; succinate:(acceptor) oxidoreductase

2. Extra-mitochondrial (wholly extra-mitochondrial or with an extra-mitochondrial component)

LD	: lactate dehydrogenase ; 1.1.1.27 ; L-lactate:NAD oxidoreductase
HBD	: 2-hydroxybutyrate dehydrogenase ; isoenzyme of 1.1.1.27 ; DL-2-hydroxybutyrate:NAD oxidoreductase
α -GPD-NAD	: glycerol-3-phosphate dehydrogenase ; 1.1.1.8 ; L-glycerol-3-phosphate:NAD oxidoreductase
β -HBD	: 3-hydroxybutyrate dehydrogenase ; 1.1.1.30 ; D-3-hydroxybutyrate:NAD oxidoreductase
MD	: malate dehydrogenase ; 1.1.1.37 ; L-malate:NAD oxidoreductase
GPD	: glyceraldehydephosphate dehydrogenase, triosephosphate dehydrogenase ; 1.2.1.12 ; D-glyceraldehyde-3-phosphate:NAD oxidoreductase

3. Pentose phosphate shunt

G6PD : glucose-6-phosphate dehydrogenase ; 1.1.1.49 ;
D-glucose-6-phosphate:NAD oxidoreductase

6PGD : phosphogluconate dehydrogenase ; 1.1.1.43 ;
6-phospho-D-gluconate:NAD(P) oxidoreductase

ENZYME CONTROLS

- c : control enzyme activity in the absence of added
exogenous substrate or co-enzyme
- c-NAD : control enzyme activity in the absence of added
exogenous substrate but in the presence of added
NAD
- c-NADP : control enzyme activity in the absence of added
exogenous substrate but in the presence of added
NADP

MISCELLANEOUS

- ADP : adenosine diphosphate
- ATP : adenosine triphosphate
- $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$: calcium chloride dihydrate
- $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$: cobaltous chloride
- EDTA : ethylene diamine tetra-acetate, dipotassium salt
- PHA : phytohaemagglutinin
- PVA : polyvinyl alcohol
- PVP : polyvinyl pyrrolidone

TETRAZOLIUM SALTS

BT : 2, 2', 5, 5'-tetraphenyl-3, 3'-(3,3'-dimethoxy-
4,4'-diphenylene) ditetrazolium chloride.

MTT/

- MTT : 3-(4,5-dimethyl thiazolyl-2)-2,4-diphenyl
tetrazolium bromide
- NBT : 2,2'-di-p-nitrophenyl-5,5'-diphenyl-3,3'-(3,3'-
dimethoxy-4,4'-diphenylene ditetrazolium chloride)
- NT : 2,2'-(p-diphenylene-bis-(3,5-diphenyl) ditetrazolium
chloride

**INTRODUCTION : RATIONALE FOR THESE STUDIES AND
LAY-OUT OF TEXT**

In 1966, when this study was first contemplated, there was considerable interest in reported elevations of serum lactate dehydrogenase levels in patients with megaloblastic anaemia. It was suggested, on the basis of biochemical estimations of bone marrow homogenates, that the marrow might be the source of increased enzyme release consequent upon the intramedullary destruction of megaloblasts. Increased serum lactate dehydrogenase levels had also been described in acute leukaemia and there was suggestive evidence, from a few cytochemical studies, of increased dehydrogenase activity in blast cells. In view of the marrow cell maturation arrest which is a feature of both megaloblastosis and acute leukaemia it was considered to be of potential value to study, cytochemically, the activity of lactate and other extra- and intra-mitochondrial dehydrogenases in normal and leukaemic marrow cells in relation to their stage of maturation.

Comparatively little is known of the shuttle systems in mammalian cells which transfer hydrogen ions, generated in the extramitochondrial Embden-Meyerhof glycolytic pathway, through the mitochondrial membrane, to the intra-mitochondrial respiratory chain. A number of studies of solid/

solid tumour homogenates have suggested that some shuttle system enzymes are selectively deleted in malignant cells and that this results in increased glycolytic enzyme activity with an increase in cell lactate dehydrogenase content. This abnormality was suggested as an explanation for the Warburg (1930) hypothesis that malignant cells show an obligatory aerobic glycolysis because they are unable to utilise the respiratory pathway. Since it is difficult to assess the significance of biochemical assays based on the mixed cell population of tumour homogenates it was decided to extend the cytochemical study to include shuttle system dehydrogenases, and also a transhydrogenase, and relate their activity to cellular maturation and leukaemic change.

The reported enzyme deletions in solid tumours have been shown not to result from enzyme inhibition. A lactate dehydrogenase inhibitor has, however, been reported in the plasma of patients with chronic renal failure and, since glycolytic enzyme inhibition might adversely affect erythroblast maturation and/or red cell survival, a cytochemical study of the activity of this enzyme was made in erythroid cells in patients with chronic renal failure.

The proposed studies were hampered by the limitations of existing cytochemical methods and a large part of the experimental/

experimental work was therefore devoted to the development of new cytochemical techniques. This involved a consideration of the potential disadvantages of cell fixatives which may completely inhibit weakly reacting dehydrogenases and also, by a lipid-clearing effect, affect the permeability of the lipo-protein mitochondrial membrane. The application of polyvinyl alcohol, a collagen polypeptide and an inert sucrose polymer (Ficoll), as cell protective agents during incubation for cytochemical reactions, has also been investigated. A cold-ethanol fixation technique incorporating polyvinyl alcohol in the incubation solution was found suitable for a study of erythroblasts, but the relatively fragile blast cell and the weakly-reacting lymphocyte and polymorph posed a more rigorous test for enzyme cytochemistry. A second cytochemical technique, which did not require cell fixation and in which the reaction end-product was measured by means of scanning and integrating microdensitometry, was therefore developed for a study of the latter cells. The experimental methods and principles involved have been discussed in some detail since there is no equivalent published text devoted to dehydrogenase enzyme cytochemistry of marrow and blood cells.

The finding, in this study, of relatively low intramitochondrial/

intra-mitochondrial and α -glycerolphosphate mitochondrial membrane shuttle system enzyme activity in the leukaemic lymphoblast and the EB2 cell suggested that these cells might be metabolically dependent on extra-mitochondrial glycolysis and therefore susceptible to glycolytic enzyme inhibition. This hypothesis was therefore tested in a cell culture system designed to determine the in vitro cytotoxic effect of sodium oxamate (a lactate dehydrogenase inhibitor) at physiological osmolality.

The introduction, experimental methods, results and discussion for each section have been presented separately although the common principles of enzyme cytochemistry are discussed in Part I. The main conclusions and the potential value of the observations are discussed briefly in a concluding section.

The abbreviations used in the text are adopted from the 'Instructions to Authors' of the British Journal of Haematology. Other abbreviations are listed in a separate section, for ease of reference, together with the appropriate enzyme nomenclature according to the Recommendations (1964) of the International Union of Biochemistry.

PART I : PRINCIPLES OF CYTOCHEMICAL TECHNIQUES
AS APPLIED TO THESE STUDIES

1. Choice of Anticoagulant and Preparation of Films

In order to study several different enzymes, with equivalent controls, in a single marrow aspirate, and also to obtain sufficient films for diagnostic purposes, it was found necessary to take the aspirate into anticoagulant and spread the desired number of films in the laboratory at leisure. This also allowed more uniform films of approximately equal cellular density to be made. Fairly dilute films were made in order to avoid cell clumps; this facilitated the recognition of cells, which may be difficult when only a nuclear counterstain is used, and also aided subsequent scoring of the enzyme reaction, particularly when the microdensitometer was used.

Marrow films were spread by hand usually within an hour of aspiration; when a longer delay was envisaged (maximum 24 hours) the anticoagulated specimen was stored at $0-4^{\circ}\text{C}$. Dried EDTA (sequestrene) in a 2.5 ml plastic container (Stayne Laboratories Ltd.) was found to be the anticoagulant of choice. There was no demonstrable loss of LD activity in films made from the anticoagulated specimen after storage at $0-4^{\circ}\text{C}$ for 24 hours, compared with films made at the time of aspiration. Melnick (1967) also found EDTA to be non-inhibitory to dehydrogenase enzymes and Elliott, Jepson and Wilkinson (1962) found no difference between/

between the serum LD activity and the plasma LD activity obtained from blood anticoagulated with EDTA. Bohn (1965) incorporated EDTA in the buffer of his incubation solutions in order to avoid the theoretical risk that certain heavy metals may inactivate the sulphhydryl groups of dehydrogenases, and he also found no inhibition of enzyme activity. Lehninger (1965) reported that EDTA, and other metal-chelating agents, block mitochondrial swelling in cells and this possible protective function of EDTA also favoured the choice of this anticoagulant.

2. Principles of Cell Fixation

If an unfixed marrow or blood film is incubated at 37°C in an aqueous reaction mixture at near neutral pH, then the majority of cells will disrupt and be removed from the slide. The object of fixation is to impose a degree of denaturation on the cell protoplasm so that the cells do not disrupt, nor their soluble constituents escape into the reaction solution during incubation. Fixation, however, has a number of disadvantages. It affects the lipo-protein bonds which stabilise membranes and which may control enzymatic activity (Wolman, 1955; Barka and Anderson, 1963; Danielli, 1953); as a consequence, all cell membranes become permeable and there is a theoretical risk/

risk of loss of soluble enzymes from the cell. Fixation may also inhibit an enzyme or alter its physical properties or physical distribution within the cell. Partial enzyme inhibition is particularly not desirable for the cytochemical demonstration of weakly reacting dehydrogenases - if a tetrazolium salt is used as the hydrogen capture agent, then its rate of reduction and of formazan production may not be high enough for the latter to precipitate within the cell and a false negative reaction will result. Also, if its rate of production is slow the formazan may follow a diffusion gradient to an absorbing site, such as a lipid droplet, and give rise to false localisation.

The introduction of 60% acetone as a fixative for dehydrogenase cytochemistry in marrow and blood cells (Quaglino and Hayhoe, 1960) was a significant advance since it provided reasonably good fixation without gross enzyme inactivation. It does, however, result in significant enzyme inactivation (Libnoch, Yakulis and Heller, 1966; Gough and Elves, 1967) and this becomes particularly important in the more weakly reacting mature myeloid cells. While de Souza and Kothare (1959) were able to demonstrate SD activity in polymorphs using a supravital technique, and Balogh and Cohen (1961) also found oxidative activity in polymorphs/

polymorphs using unfixed smears, Quaglin~~o~~ and Hayhoe (1960) and Hayhoe, Quaglino and Doll (1964) invariably obtained no reaction for SD in these cells after acetone fixation, and even the strongly reacting LD showed only a few isolated granules in a small proportion of cells.

Kaplow and Burstone (1963) recommended the use of 60% acetone buffered to pH 4.2 with citrate, on the basis that this achieved better cellular preservation and also greater demonstrable activity of some enzymes. Melnick (1968), in turn, favoured fixation for 60 sec with 100% acetone at 0°C and claimed that this prevented the loss of soluble enzymes from the cell which he stated was a disadvantage with 60% acetone fixation. His technique, however, required an 18 hour incubation period in order to achieve good formazan production and this implies a significant degree of enzyme inactivation. Libnoch et al. (1966) also found that fixation with 60% acetone resulted in enzyme inhibition and they instead fixed for 5 sec using a formol-calcium solution. This technique, however, required an additional hydrogen acceptor (phenazine metho-sulphate), as well as the tetrazole, in order to demonstrate LD activity and this again implies enzyme inactivation.

In view of these statements of the inadequacies of existing fixation techniques part of this study was devoted/

devoted to the investigation of alternative methods of fixation and also to the use of 'no-fix' techniques.

3. Use of 'Protective' Incubation Media

When fresh cryostat sections are placed in glycylglycine buffer, then 50-60% of the nitrogenous material of the section passes into solution (Altmann and Chayen, 1965). This can be prevented if a high concentration (20% w/v) of the colloid stabiliser polyvinyl alcohol (PVA) is added to the incubation solution (Altmann and Chayen, 1965) or, alternatively, if a 30% w/v concentration of various collagen-derived polypeptides (Altmann and Chayen, 1966) is used. The soluble enzymes are also retained within the section in addition to the nitrogenous matter.

When intact, undamaged cells in free suspension in an incubation solution are studied, the cell membrane remains relatively impermeable and there is clearly less of a tendency for dissolution of unfixed protoplasm. Ackerman (1960) and Melnick (1968) in fact tried to increase cell permeability to exogenous substrate by treating the membrane with Renex 20, trypsin, pepsin and chymotrypsin, but this resulted in excessive damage to the cells.

When intact cells are instead smeared on to glass slides/

slides, air dried and not fixed, or only partially fixed, before immersion in an incubation solution, then some additive requires to be added to the solution to retain cells on the slide and also to prevent soluble enzymes from diffusing through the damaged cell membrane. Bohn (1965) incorporated a 1.2% w/v solution of agar in the incubation medium, but this resulted in a variable reaction intensity and difficulty in the recognition of cells (Bohn, 1970). Libnoch et al. (1966) used a 2.5% w/v final concentration of gelatine in phosphate buffer, while Melnick (1968) immersed acetone-fixed films in a 5% w/v concentration of polyvinyl pyrrolidone (PVP) in saline prior to incubation.

These low concentrations of additives have been used, with limited success, in enzyme cytochemistry for many years, but the more recent beneficial results claimed for higher concentrations in histochemistry (Altmann and Chayen, 1965; 1966) appeared to justify a reappraisal of the value of these additives in dehydrogenase cytochemistry.

4. Endogenous (Control) Enzyme Activity

Formazan production by a dehydrogenase enzyme, at a pH of less than 8.0, in the absence of added exogenous substrate must be presumed to represent the activity of enzymes/

enzymes acting on endogenous substrate retained within the cell; considerably stronger enzyme activity should be obtained in the presence of added exogenous substrate (de Souza and Kothare, 1959). The actual amount of endogenous substrate remaining within a cell at the time of incubation will depend in part on the previous metabolic activity of the cell but will be influenced considerably by the degree of permeability of the cell membrane. It should, however, be possible to achieve a state of increased membrane permeability so that endogenous substrate can leak out, and exogenous substrate enter in, without corresponding enzyme loss due to the colloidal nature, molecular size and tissue binding of most dehydrogenases (Dixon and Webb, 1964).

When a fixative is used, the resulting increase in membrane permeability, together with a degree of enzyme inhibition, is usually sufficient to give near-negative endogenous (control) activity, and control preparations are used merely as a check against processing errors. When unfixed smeared cells, or intact cells in suspension, are studied, then there is usually significant positivity in the control specimens. This represents non-specific enzyme activity in the sense that several substrates may be involved, and its value requires to be subtracted from the gross enzyme/

enzyme activity obtained in the presence of added specific substrate to give net, specific, exogenous enzyme activity.

Attempts have been made to dialyse out endogenous substrate in order to achieve negative controls. Wagner, Meyerriecks and Sparaco (1956) were able to reduce, almost to zero, the endogenous oxygen consumption of leucocyte suspensions by dialysis against distilled water, and subsequently restore it again by the addition of appropriate substrate. Dhungat and Sreenivasan (1954) and Cohen (1962) similarly achieved negative controls for histochemical enzyme reactions by rinsing in pyrophosphate and phosphate buffers respectively. Gough and Elves (1967) used an isotonic saline rinse to obtain negative controls in unfixed marrow films.

5. Choice of Tetrazolium Salt

The dehydrogenase enzymes oxidise their substrate by removing hydrogen and passing it, in most cases, to an appropriate co-enzyme, which becomes reduced. This, in turn, acts as the hydrogen donor for the next stage in the hydrogen transport system to allow the oxidised form of the co-enzyme to be regenerated. The function of a tetrazolium salt is to act as an alternative hydrogen acceptor/

acceptor at this stage. These colourless, or pale coloured, heterocyclic compounds are readily reduced to form a dark-coloured insoluble formazan which is precipitated within the cell to form the reaction end-product.

The earlier monotetrazolium salts gave unsatisfactory end-products and it was not until the introduction of the ditetrazolium salt NT (Antopol, Glaubach and Goldman, 1948), and its methoxy derivative BT (Rutenburg, Gofstein and Seligman, 1950), that satisfactory results were obtained. More recently, MTT (Pearse, 1957) and NBT (Tsou, Cheng, Nachlas and Seligman, 1956) have been introduced and NBT is now widely used on account of its higher redox potential, lower lipid solubility and higher substantivity (affinity) for protein which gives accurate intracellular localization. Prior to the introduction of ditetrazoles of high redox potential it was suggested by Farber, Sternberg and Dunlap (1956) that tetrazolium salts were only capable of demonstrating NADH_2 and NADPH_2 diaphorases, rather than individual dehydrogenases, but this was subsequently shown to be erroneous (Nachlas, Walker and Seligman, 1958; Hess, Scarpelli and Pearse, 1958a).

The advantages of NBT as an efficient hydrogen acceptor do/

do not necessarily make this the tetrazole of first choice in all histochemical and cytochemical studies. A relatively high lipid solubility of a tetrazole may be of value if it assists penetration of the salt through lipoprotein barriers such as the mitochondrial membrane, and NT has the advantage that its formazan may be eluted from the tissue under study using 10% heptanol in tetrachlorethane (Jones, Maple, Aves, Chayen and Cunningham, 1963) so that a quantitative measure of the dehydrogenase activity of a tissue can be made by spectrophotometry. Thus it is now possible to select the tetrazolium salt most suitable for the cell or tissue to be studied and the method of quantitation to be used. The elution technique described for NT is not suitable, however, for a cytochemical study of a pleomorphic tissue such as the marrow, since the crystalline nature of its formazan results in an inhomogeneous distribution of high extinction which is unlikely to be capable of precise measurement either by visual scoring methods or by microdensitometry (Chayen and Denby, 1968). Both MTT and NBT are, however, suitable for cytochemical methods and the factors dictating the choice of these tetrazoles in these studies are given in the appropriate sections.

It/

It has already been confirmed that the amount of formazan deposited within a tissue undergoing a histochemical dehydrogenase reaction (for succinate dehydrogenase) is proportional to the amount of enzyme present (Jones et al., 1963).

6. Use of Metallic Ion Activators and Menadione

When a tetrazolium salt of relatively low redox potential is used (e.g. MTT) the formazan produced tends to aggregate and crystallise, giving rise to poor localisation and difficulty in visual scoring. Certain metallic ions (Al^{3+} , Co^{2+} , Cu^{2+} , Mg^{2+} and Mn^{2+}) form metallic complex 'salts' with formazans and the resulting capture reaction prevents this crystallization.

These metallic ions have also been used by some investigators as activators of dehydrogenases (de Souza and Kothare, 1959; Barka and Anderson, 1963). Rutenburg, Wolman and Seligman (1953) showed that the reaction rate for succinate dehydrogenase (SD), using BT as the tetrazole, could be greatly increased by the addition of Ca^{2+} , Mg^{2+} , Al^{3+} , and also bicarbonate, to the reaction mixture. When MTT is used instead, then Al^{3+} will increase demonstrable SD activity when the tissue is weak in this enzyme (Pearse, 1960), but when NBT is used these additives are not required (Nachlas, Tsou, de Souza, Cheng and/

and Seligman, 1957) .

Wattenberg and Leong (1960) observed that the incorporation of the quinone, menadione, in the incubation solution as an additional electron carrier increased SD activity in tissue sections, and this compensated for partial enzyme inhibition during acetone fixation.

Ogawa and Hashimoto (1961) and Ackerman (1964) also reported enhanced SD activity in the presence of menadione, while Blanchaer, van Wijhe and Mozersky (1963) observed activation of intra-mitochondrial α -glycerolphosphate dehydrogenase (α -GPD) .

7. Reaction pH and 'Nothing' Dehydrogenase

In dehydrogenase cytochemistry it is important to keep the pH of the incubation mixture below 8.0 to prevent non-specific reduction of the tetrazolium salt, the so-called 'nothing' dehydrogenase reaction. This may result either from the action of alcohol dehydrogenase or from the effect of sulphhydryl groups (Seligman and Rutenburg, 1951) . For this reason the majority of workers have used a pH of 7.4-7.5 for dehydrogenase cytochemistry, irrespective of the enzyme under study. At a pH of greater than 7.4 considerable mitochondrial swelling occurs and this alters the oxidation velocity of various substrates, such as succinate/

succinate (Pearse, 1960). Thus a neutral pH has the advantage of maintaining mitochondrial structure (Scarpelli and Pearse, 1958) and it also ensures co-enzyme stability since NAD and NADP are rapidly destroyed at alkaline pH (Kaplan, Colowick and Carr Barnes, 1951).

8. Respiratory Pathway Blockade

Since molecular oxygen and the respiratory pathway compete with the tetrazolium salt for the available hydrogen generated in the dehydrogenase reaction, attempts have been made to block the electron transport chain by means of azide or cyanide (Pearse, 1960) or, alternatively, to carry out the incubation in an atmosphere of nitrogen (Chayen, Bitensky, Butcher and Poulter, 1969). The introduction of the more efficient hydrogen-trapping ditetrazolium salts has largely replaced the need for respiratory blockade; these agents were therefore not used in this study although incubation was always carried out in an atmosphere of nitrogen.

9. Incubation Temperature

Tetrazolium salts undergo reduction more readily with increasing temperature above 37°C and it is necessary to ensure accurate temperature control throughout the incubation period. This was achieved in the earlier experiments of this/

this study by pre-warming the reagents and the anaerobic jar in which the slides were placed, and incubating thereafter at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$. For later experiments, a thermostatically controlled incubation box was designed which contained an inner, pre-heated incubation-humidity box which ensured adequate insulation against temperature fluctuation (see Fig. 10).

10. Co-enzyme and Substrate Concentrations

Endogenous co-enzyme levels are in general too low to allow co-enzyme dependent dehydrogenase reactions to take place without the addition of NAD or NADP (Pearse, 1960), and damage to mitochondrial and cytoplasmic membranes during incubation may theoretically lead to further loss of endogenous co-enzyme (Porter, Deming, Wright and Scott, 1953).

Hess, Scarpelli and Pearse (1958b) obtained optimal reduction of tetrazolium salt when the substrate and co-enzyme concentrations were similar at 0.1M, although good results were also obtained at a concentration of 0.01M. Wagner et al. (1956) also obtained an increase in the activity of α -GPD and SD up to a substrate concentration of 0.08M. These concentrations were higher than those of earlier workers but it would seem important to maintain high substrate and co-enzyme concentrations in cytochemical studies/

studies since limited membrane permeability may restrict their entry into the cell; it is particularly important to avoid co-enzyme exhaustion which would become rate-limiting for the enzyme reaction. Only in certain circumstances - at low concentration under controlled conditions - does the velocity of an enzyme reaction parallel the substrate concentration and above a certain minimal substrate concentration there is no further increase. Provided that sufficient substrate is present to overcome this variability at low concentration and provided that excess, which might inhibit the reaction, is also avoided, then the actual concentration used would not appear to be critical.

11. Morphological Recognition of Cells Following Incubation

It is an essential pre-requisite of cytochemistry that it should be possible to recognise individual cells at the completion of the incubation procedure. This is dependent on cell preservation and the counterstain used. Since the formazan produced in dehydrogenase reactions usually consists of small granules and/or a diffuse background reaction within the cytoplasm, it is not possible to use a cytoplasmic counterstain since this will obscure the diffuse reaction. Of three widely used nuclear counter-stains/

counterstains - Carrazi's haematoxylin, nuclear fast red (or safranin) and methyl green - the haematoxylin was found to outline nuclear chromatin in more detail and allow easier differentiation between, for example, late erythroblasts and lymphocytes. For this reason it was used for the cytochemical study of erythroblasts at their various stages of maturation.

Methyl green was found to be a more suitable counter-stain for the cytochemical study of leucocytes where microdensitometry, with readings taken in green light, was used to quantitate formazan production. Small lymphocytes could be differentiated from erythroblasts by the unstained nuclear 'halo' around the green nucleus, and more mature myeloid cells could be recognised by their nuclear shape. Where difficulty was experienced in recognising methyl green counterstained leucocytes, it was found useful to view the cell in red light to give a clear (black) outline of nuclear shape. It was, however, difficult to differentiate between myeloblasts, promyelocytes and myelocytes in a mixed population of cells and this was not attempted.

**PART II : HYDROGEN TRANSPORT SYSTEMS
IN MEGALOBLASTS**

1. Introduction

The lactate dehydrogenase (LD) level in human serum is pathologically raised in a number of haematological conditions including haemolytic anaemia (Zimmerman and Weinstein, 1956) and megaloblastic anaemia secondary to deficiency of both vitamin B₁₂ (Hess and Gehm, 1955; Heller, Weinstein, West and Zimmerman, 1960a) and folic acid (Gordin and Enari, 1959). Exceptionally high levels are, however, found in megaloblastic anaemia - from 5-21 times the upper limit of normal (Hess and Gehm, 1955) - and these levels are not matched by comparable increases in haemolytic anaemia unless there is associated megaloblastic change (Gronvall, 1961).

The mature erythrocyte in megaloblastic anaemia also shows an increased content of LD which may be as high as 50-60% above normal (Emerson, Withycombe and Wilkinson, 1967). The degree of haemolysis which occurs in megaloblastic anaemia is, however, insufficient to explain the gross elevations in serum LD, even when the increased LD content of the red cell is taken into account, and some additional source of LD release is therefore likely.

Homogenates of megaloblastic marrow have been shown to contain a 2-3 fold increase in LD activity per mg of protein compared with similarly prepared homogenates of normoblastic/

normoblastic marrow (Heller et al., 1966b; Elliott and Fleming, 1965) and thus marrow tissue is a potential source of origin of the elevated serum LD levels. This is supported by the similar LD isoenzyme pattern of the serum, red cells and marrow of megaloblastic anaemia patients and which differs from the isoenzyme pattern found in other tissues such as liver and muscle (Emerson et al., 1967).

The intramedullary cell death and ineffective erythropoiesis of megaloblastic anaemia is well known (Finch, Coleman, Motulsky, Donohue and Reiff, 1956). This is associated with a failure of DNA synthesis and with an arrest of early polychromatic megaloblasts in the G2 phase of the mitotic cycle (Wickramasinghe, Chalmers and Cooper, 1967). Cell death probably occurs at the early polychromatic stage of maturation. If individual megaloblasts contain an increased amount of LD then this, in combination with substantial intramedullary cell disruption, would contribute significantly to the increased serum enzyme level.

It cannot be assumed from homogenate studies that individual megaloblasts have an increased LD content. If a homogenate of a megaloblastic marrow is compared with a homogenate of a marrow showing normoblastic erythropoiesis (even with an equivalent degree of erythroid hyperplasia) the/

the maturation arrest of megaloblastosis will ensure a higher content of early and intermediate erythroblasts in the former. Since the LD content of developing erythroid cells decreases progressively with maturation then the homogenate of the megaloblastic marrow will always show higher activity. Since this question can only be resolved by cytochemical techniques a study was made of the LD content of individual megaloblasts compared with normoblasts at equivalent stages of maturation and under approximately similar degrees of erythroid hyperplasia.

Heller et al. (1960b) found that megaloblastic marrow homogenates also showed increased activity of pentose phosphate shunt and tricarboxylic acid cycle dehydrogenases, and the cytochemical study was therefore extended to include representative enzymes from each pathway. Sacktor and Dick (1960) in a study of mouse leukaemia reported a decrease in activity of an α -glycerolphosphate shuttle system operating between the extramitochondrial Embden-Meyerhof pathway and the intramitochondrial respiratory pathway (see Fig. 4), and since the hyperplasia and maturation arrest of megaloblastic erythropoiesis shows some similarities to that of acute leukaemic transformation the activity of two enzymes from this shuttle system was also studied.

2. Development of Method

2.1 Preparation of films Bone marrow specimens were taken into EDTA and fairly dilute marrow films spread as previously described (see I.1). The films were then allowed to air dry at room temperature for at least 60 min prior to fixation.

2.2 Fixation The use of 60% acetone for 30-60 sec at room temperature (Quaglino and Hayhoe, 1960) was a significant advance in fixation for dehydrogenase cytochemistry of marrow cells, and this technique was therefore used as a basis for comparison with other methods. Fixation for 30 sec, rather than 60 sec, at room temperature resulted in a stronger reaction with only slight deterioration in cell preservation, but fixation with 60% acetone at 0-4°C gave significantly poorer preservation. Fixation with 100% acetone for 60 sec at 20°C, and also at 0°C as subsequently used by Melnick (1968), resulted in a weaker reaction; the use of 60% acetone buffered to pH 4.2 (Kaplou and Burstone, 1963) seemed to give identical results to those of the Quaglino and Hayhoe (1960) method.

The latter method, however, resulted in poor reactions for the less active enzymes, particularly in late erythroblasts. There was also some variation in demonstrable enzyme/

enzyme activity from day to day which appeared to be associated with inadequate cell preservation and loss of cytoplasm in the late erythroblasts. Better results were obtained when the marrow films were allowed to air dry for at least 60 min prior to fixation and when the acetone was blotted dry, rather than washed off the slide, at the end of fixation, but the day-to-day variation was still unsatisfactory. The use of formalin fixation as an alternative (Libnoch et al., 1966) was less satisfactory: considerable enzyme loss, or inhibition, occurred and it was found difficult to standardise the fixation technique due to the very short (5 sec period) recommended. More prolonged formalin fixation resulted in complete inhibition of all demonstrable enzyme activity.

When no fixative was used the majority of cells were lost from the slide during the subsequent incubation. When PVA, at a concentration of 150 mg/ml, was added to the incubation mixture as a cell preservative (see I.3), then considerably more cells were retained on the slide, but cell preservation was still poor and the presence of extracellular reaction end-product confirmed leakage from the cells of either enzyme or formazan. A good reaction product was obtained, however, in those cells which were retained on the slide in an apparently intact form. It was/

was not possible to substantially increase the PVA concentration, on account of increasing viscosity, and it was therefore necessary to use a fixative in addition to the PVA to achieve adequate cell preservation. The combination of fixation in 100% ethanol at -70°C for 30 sec together with incubation in the presence of PVA gave best results. This achieved as good formazan production as acetone fixation for the strongly reactive enzymes, but gave more consistent results on a day-to-day basis and allowed a stronger reaction in late erythroblasts associated with better cellular preservation. Cold ethanol fixation without the subsequent use of PVA in the incubation solution was less satisfactory than the Quaglini and Hayhoe (1960) method due to poor preservation of cells.

A carefully standardised technique was used. Air dried slides were held by rubber-tipped forceps in absolute ethanol at -70°C in a vacuum flask. This degree of chilling was achieved by means of solid carbon dioxide and was checked by a low-reading thermometer. After fixation for exactly 30 sec the slides were dried very rapidly by shaking in air, then immersed almost immediately in incubation solution prepared during the period of 60 min allocated for air drying of the films.

Fixation with 100% ethanol at $0-4^{\circ}\text{C}$, and at 20°C , resulted/

resulted in almost complete enzyme inhibition, and super-cooling of the fixative therefore appeared to be critical. Super-cooling of marrow fragments, without ethanol fixation, followed by sectioning in a cryostat at an ambient temperature of -30°C , with the knife cooled to approximately -70°C , was not successful, due to cellular disruption.

Super-cooling of cells does not appear to adversely affect dehydrogenase enzyme activity. Burstone (1964) achieved satisfactory demonstration of intramitochondrial dehydrogenases in unfixed, frozen sections and cell structure such as mitochondria seem fairly resistant to freezing and thawing. Melnick (1968) subsequently showed that marrow cells may be stored at -85°C for long periods without perceptible change in dehydrogenase enzyme activity. Thus the enzyme inhibition which does occur with cold ethanol fixation is presumably due to the action of the ethanol.

The addition to the incubation solution of other cell protective agents - PVP (Pearse, 1960; Hayhoe *et al.*, 1964; Melnick, 1968) and gelatine (Libnoch *et al.*, 1966) - was less satisfactory, at the concentrations used by these authors, than PVA at a final concentration of 150 mg/ml.

2.3 Incubation procedure In order to prevent competition between/

between the tetrazole and atmospheric oxygen for the hydrogen generated by the dehydrogenase, all incubation reactions were performed in an atmosphere of nitrogen in a pre-warmed McIntosh and Fildes anaerobic jar. In the earlier experiments the marrow films were covered with incubation solution, sealed with a coverslip, and placed in a Petri dish on moist filter paper to avoid evaporation and concentration of reagents. In later experiments, and in the study of megaloblastic marrows, a perspex tray comprising a series of troughs, each designed to hold one slide flooded with incubation solution, and covered with a perspex lid to prevent evaporation, was used.

The duration of incubation was chosen to give an adequate number of formazan granules to facilitate scoring - this ranged from 30 min for the stronger reacting enzymes to 120 min for the weakly reacting dehydrogenases. At the end of the incubation period the slides were removed from the troughs, rinsed gently in tap water to remove remaining incubation solution which might prolong the reaction, then counterstained with Carrazi's haematoxylin for 5 min. The slides were then stored in the dark without applying immersion oil and scored within 48 hours. There was no apparent reduction in the quantity of formazan/

formazan per cell over a period of one week when stored in this way.

2.4 Composition of incubation solutions The composition of the incubation solutions used for the demonstration of individual enzymes is given in Table I. The tetrazolium salt MTT, with added CoCl_2 to prevent formazan crystallization, was chosen in favour of NBT since it gave a negative reaction in the control preparations lacking substrate and since the granular nature of the MTT-formazan, without significant diffusion, facilitated visual scoring. The use of the metallic ion activator MgCl_2 and the addition of sodium bicarbonate and menadione was adopted from the methods of previous workers although a critical evaluation of their effect in increasing formazan production was not made.

2.5 Incubation pH This was maintained between 7.0 and 7.4 since there was no detectable difference in formazan production within this pH range.

2.6 Co-enzyme concentrations The addition of NAD and NADP, at final concentrations of 0.5 mg/ml, 1 mg/ml and 5 mg/ml, was studied for incubation periods of 13, 30 and 90 min for the enzymes LD and G6PD. There was no detectable increase in/

TABLE I

Composition of Incubation Solutions and Duration of Incubation for Cold-ethanol Fixation Technique

	LD	α -GPD-NAD	MD	α -GPD	SD	G6PD	6PGD
Duration of incubation (min)	60	90	30	90	90	120	120
Substrate*	0.2 ml	37.8 mg	37.4 mg	37.8 mg	54.0 mg	18.0 mg	5.8 mg
MTT (mg)	1.0	2.0	1.0	2.0	2.0	2.0	2.0
NAD (mg)	1.0	2.0	1.0	-	-	-	-
NADP (mg)	-	-	-	-	-	4.0	4.0
Distilled water (ml)	1.0	1.0	1.0	1.0	1.0	1.0	1.0
PVA - 300 mg/ml of 0.2M Tris buffer pH 7.4 (ml)	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Cobaltous chloride 0.05M (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Magnesium chloride 0.05M (ml)	-	-	-	-	-	0.1	0.1
Aluminium chloride 0.01M (ml)	-	-	-	0.1	0.1	-	-
Sodium bicarbonate 0.6M (ml)	-	-	-	0.1	0.1	-	-
Menadione, sodium bisulphite - 4 mg/ml dist. water (ml)	-	-	-	0.1	0.1	-	-

* see 'source list of chemicals and equipment'

in formazan production at the higher co-enzyme concentrations and a final concentration of 0.5 mg/ml was therefore adopted.

2.7 Substrate concentrations The substrate concentrations used were based on those of de Souza and Kothare (1959); Pearse (1960); Balogh and Cohen (1961) and Hayhoe et al. (1964) and are shown in Table I.

2.8 Negative control (endogenous) enzyme activity A control film, incubated in the presence of MTT but in the absence of added specific substrate or co-enzyme, was included with each marrow as a check against processing errors. This control film showed either no formazan production, or only the occasional formazan granule, throughout the period of study.

2.9 Semi-quantitative visual scoring technique Both the number and size of formazan granules increased with duration of incubation and it was therefore assumed that an increase in granule size and number represented an increase in enzyme activity. A semi-quantitative visual scoring technique based on granule size and number was therefore devised to give a more objective assessment of enzyme activity than the visual impression (see Table II).

Twenty-five/

TABLE IISemi-quantitative Visual Scoring Method

TYPE OF FORMAZAN GRANULE	GRANULE NUMBER	ARBITRARY SCORE
Fine	0	0
	1-10	1
	11-20	2
	20	3
Moderately coarse	1-5	4
	6-10	5
	10	6
Coarse	1-5	7
	6-10	8
	10	9

Twenty-five, 33, or occasionally 50, erythroblasts were scored at each of the early, intermediate and late stages of erythroid maturation and the scores adjusted to a mean figure for 100 cells. The reproducibility of the scoring method and the incubation technique was assessed by carrying out five separate estimations for each enzyme on a single marrow specimen using different incubation solutions (see Table III).

3. Patients Studied and Results

Unlike Libnoch et al. (1966), who failed to demonstrate an increase in LD activity in megaloblasts, it was felt necessary in this study to match the degree of erythroid hyperplasia in the normoblastic and megaloblastic marrows and to compare enzyme activity at equivalent stages of maturation.

Marrow films from 5 patients with severe vitamin B₁₂ deficiency and showing well developed megaloblastic change (see Table IV) were compared with five marrow films showing normoblastic hyperplasia due to secondary polycythaemia or haemolytic anaemia. The corresponding mean percentage of erythroblasts in the two groups was 46.8% and 50.5% respectively. The mean scores for erythroblasts at the early and intermediate stages of maturation/

TABLE III

Reproducibility of Cold-ethanol Fixation Technique
Using a Semi-quantitative Visual Scoring Method
(Mean and Standard Deviation for
Five Separate Estimations)

LD	α -GPD-NAD	MD	α -GPD	SD	G6PD	6PGD
381	35	310	58	65	154	81
± 35	± 8	± 32	± 11	± 16	± 25	± 19

TABLE IV

Laboratory Details for Five Patients Showing Frank Megaloblastic Erythropoiesis

MARROW ERYTHROBLASTS %	MORPHOLOGICAL CRITERIA					SERUM B ₁₂ LEVEL (pg/ml)	SERUM FOLATE LEVEL (ng/ml)	CLINICAL DIAGNOSIS
	METAMYELOCYTES (% GIANT FORMS)	ERYTHROBLASTS, % SHOWING						
		NUCLEAR CYTOPLASMIC ASYNCHRONY	HOWELL- JOLLY BODIES	NUCLEAR FRAGMENTA- TION				
40.2	52	26	0	5	55	3.0	Pernicious anaemia	
50.7	39	52	7	4	45	6.8	Pernicious anaemia	
60.5	54	35	4	6	55	2.8	Nutritional deficiency	
45.5	49	51	4	5	55	4.6	Pernicious anaemia	
37.0	50	60	11	7	5	8.8	Pernicious anaemia	

maturation were significantly ($P < 0.05$) higher for megaloblasts compared with normoblasts (see Tables V and VI). In late megaloblasts, only the intra-mitochondrial enzymes showed a significant increase (see Table VII); but there was evidence of a dual population with some late megaloblasts, and even macrocytes, (see Fig. 1), showing an obvious increase in both intra- and extra-mitochondrial enzymes while other cells did not. This did not appear to be due to variation in cellular preservation or to the retention or loss of cytoplasm as experienced earlier with acetone fixation.

Additional marrows from seven patients in the remission stage of acute lymphoblastic leukaemia and showing slight megaloblastic change (see Table VIII) due to maintenance chemotherapy with methotrexate were also studied and compared in the same way with seven leukaemic patients also in haematological remission but not receiving methotrexate and not showing megaloblastic erythropoiesis. The corresponding mean percentage of erythroblasts in the two groups was 31.0% and 25.9% respectively. The evidence for megaloblastic change in the patients receiving methotrexate was largely confined to giant metamyelocyte formation with little evidence of nuclear/cytoplasmic asynchrony in the erythroblasts (see Table/

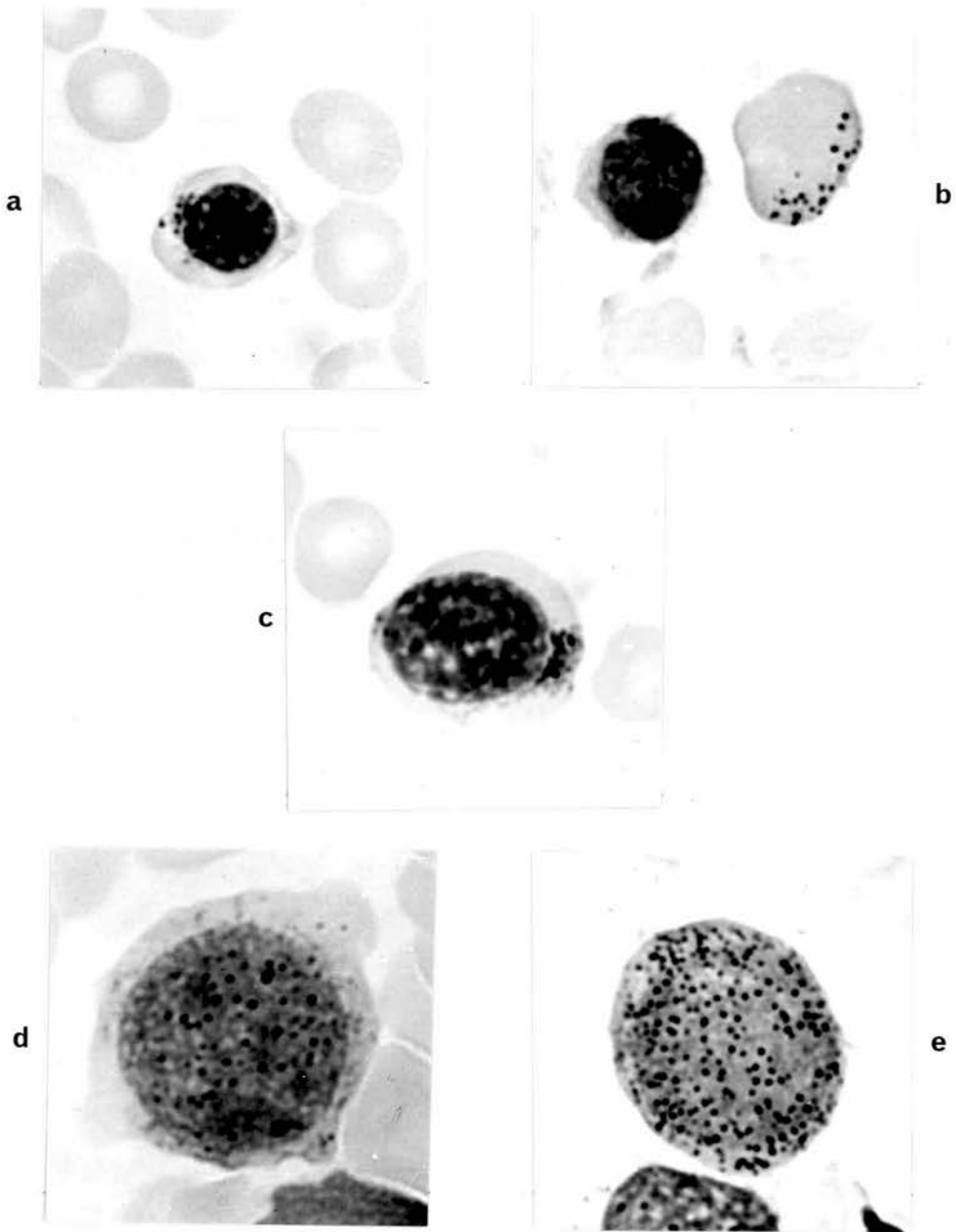


Fig. 1. Lactate dehydrogenase activity in normoblasts and megaloblasts showing increased formazan production in megaloblasts. Counterstained with Carazzi's haematoxylin. x 1600. a. late normoblast; b. macrocyte and small lymphocyte; c. intermediate normoblast; d. early normoblast; e. early megaloblast

TABLE V

Enzyme Scores for Early Erythroblasts

BONE MARROW MORPHOLOGY (NO. OF PATIENTS)	ENZYME SCORES (MEAN \pm STANDARD DEVIATION)						
	LD	α -GPD-NAD	MD	α -GPD	SD	G6PD	6PGD
1. Frank megaloblastic erythropoiesis (5)	704 \pm 80	299 \pm 97	668 \pm 92	441 \pm 65	495 \pm 106	544 \pm 91	374 \pm 140
2. Normoblastic hyperplasia (5)	549 \pm 79	98 \pm 27	497 \pm 89	158 \pm 51	198 \pm 48	205 \pm 81	105 \pm 19
3. Normal (no hyperplasia) (5)	547 \pm 34	25 \pm 5	340 \pm 111	20 \pm 5	44 \pm 23	373 \pm 136	153 \pm 75
4. Acute leukaemia in remission with early megaloblastic change (7)	604 \pm 44	270 \pm 62	475 \pm 106	177 \pm 71	165 \pm 56	259 \pm 71	206 \pm 48
5. Acute leukaemia in remission with normoblastic erythropoiesis (7)	583 \pm 50	245 \pm 85	526 \pm 85	170 \pm 84	148 \pm 35	342 \pm 102	260 \pm 91
Significance of difference of means (P)	1 v. 2	<0.05	<0.01	<0.05	<0.01	<0.01	<0.01
	2 v. 3	>0.1	<0.01	>0.05	<0.01	>0.05	>0.1
	4 v. 5	>0.1	>0.1	>0.1	>0.1	>0.1	>0.1

TABLE VI

ENZYME SCORES FOR INTERMEDIATE ERYTHROBLASTS

BONE MARROW MORPHOLOGY (NO. OF PATIENTS)	ENZYME SCORES (MEAN \pm STANDARD DEVIATION)						
	LD	α -GPD-NAD	MD	α -GPD	SD	G6PD	6PGD
1. Frank megaloblastic erythropoiesis (5)	701 \pm 100	222 \pm 89	529 \pm 64	290 \pm 89	443 \pm 106	536 \pm 108	297 \pm 128
2. Normoblastic hyperplasia (5)	446 \pm 64	80 \pm 26	403 \pm 61	70 \pm 36	171 \pm 80	20 \pm 8	28 \pm 7
3. Normal (no hyperplasia) (5)	384 \pm 112	25 \pm 10	338 \pm 116	21 \pm 11	48 \pm 15	229 \pm 101	205 \pm 95
4. Acute leukaemia in remission with early megaloblastic change (7)	489 \pm 75	51 \pm 44	366 \pm 87	107 \pm 60	63 \pm 24	198 \pm 115	91 \pm 46
5. Acute leukaemia in remission with normoblastic erythropoiesis (7)	445 \pm 82	165 \pm 132	367 \pm 92	49 \pm 29	60 \pm 26	380 \pm 182	200 \pm 126
Significance of difference of means (P)	1 v. 2	< 0.01	< 0.02	< 0.05	< 0.01	< 0.01	< 0.01
	2 v. 3	> 0.1	< 0.01	> 0.1	< 0.02	< 0.01	< 0.01
	4 v. 5	> 0.1	> 0.05	> 0.1	> 0.1	> 0.05	> 0.05

TABLE VII

Enzyme Scores for Late Erythroblasts

BONE MARROW MORPHOLOGY (NO. OF PATIENTS)	ENZYME SCORES (MEAN \pm STANDARD DEVIATION)						
	LD	α -GPD-NAD	MD	α -GPD	SD	G6PD	6PGD
1. Frank megaloblastic erythropoiesis (5)	447 \pm 104	73 \pm 18	289 \pm 83	171 \pm 22	245 \pm 74	284 \pm 86	208 \pm 62
2. Normoblastic hyperplasia (5)	343 \pm 94	53 \pm 2	287 \pm 91	75 \pm 29	84 \pm 33	30 \pm 8	25 \pm 5
3. Normal (no hyperplasia) (5)	363 \pm 75	20 \pm 5	251 \pm 87	50 \pm 12	30 \pm 10	156 \pm 69	58 \pm 12
4. Acute leukaemia in remission with early megaloblastic change (7)	435 \pm 89	50 \pm 21	277 \pm 70	101 \pm 57	83 \pm 39	128 \pm 60	164 \pm 58
5. Acute leukaemia in remission with normoblastic erythropoiesis (7)	387 \pm 80	40 \pm 20	338 \pm 83	50 \pm 26	64 \pm 34	215 \pm 92	150 \pm 66
Significance of difference of means (P)	1 v. 2 >0.1	2 v. 3 >0.1	4 v. 5 >0.1	>0.05 <0.01	>0.1 >0.1	>0.1 >0.05	>0.1 >0.1

TABLE VIII

Bone Marrow Details for Seven Leukaemic Patients Maintained in
Haematological Remission with Methotrexate

MARROW ERYTHROBLASTS (%)	MORPHOLOGICAL CRITERIA			
	METAMYELOCYTES (% GIANT FORMS)	ERYTHROBLASTS, % SHOWING:		
		NUCLEAR/ CYTOPLASMIC ASYNCHRONY	HOWELL- JOLLY BODIES	NUCLEAR FRAGMENTATION
29.4	31	0	3	2
13.6	12	0	3	0
40.4	34	4	3	0
55.0	12	18	2	1
15.4	40	13	1	4
27.2	15	0	1	4
35.8	14	9	0	0

Table VIII). No significant increase in enzyme activity was seen in the erythroblasts of these patients (see Tables V, VI and VII) and it is presumed that the degree of megaloblastic change was not significantly great.

When the five marrows showing normoblastic hyperplasia (mean of 50.5% normoblasts) were compared with five normal marrows (mean of 18.8% normoblasts) there was a small increase in enzyme activity at the three stages of maturation, but this was of variable statistical significance (see Tables V, VI and VII).

4. Discussion

In the only previous cytochemical study of this type, Libnoch et al. (1966) failed to show an increase in the LD content of megaloblasts compared with normoblasts. Their cytochemical technique required the action of an additional hydrogen acceptor (PMS) and a respiratory inhibitor (sodium azide) before any formazan could be generated in erythroblasts; since LD is a strongly reactive enzyme which should readily be demonstrable in developing erythroid cells this suggests either loss of enzyme by diffusion or inhibition by the formalin fixative used in some of their cases. An attempt to repeat their experiments using formalin fixation gave weak formazan production/

production and poor preservation of late erythroblasts.

The increase in activity of both intra- and extra-mitochondrial dehydrogenases in early and intermediate megaloblasts in this study is presumably a consequence of the maturation arrest and nuclear/cytoplasmic asynchrony of these cells. Although an attempt was made to match megaloblasts and normoblasts for equivalent stage of maturation, the megaloblasts were usually somewhat larger and probably represented a slightly earlier stage of maturation; despite this they showed quite a considerable increase in enzyme activity (see Fig. 1), as Emerson et al. (1967) reported for the macrocyte. The associated increase in intra-mitochondrial enzyme activity suggests that the maturation arrest results in an increased number of mitochondria relative to the apparent stage of maturation.

The mixed population of cells, in respect of enzyme activity, in late megaloblasts was not seen in late normoblasts nor in intermediate and early megaloblasts. The late megaloblasts with an apparently normal enzyme content may correspond to those cells in megaloblastic anaemia which show a minimal derangement of DNA metabolism without mitotic arrest (Menzies, Grossen, Fitzgerald and Gunz, 1966) and which may survive intramedullary destruction and pass into the blood.

The/

The majority of megaloblasts, however, undergo intramedullary death and, since these cells appear to contain a disproportionately high dehydrogenase enzyme content for their apparent stage of maturation, this is likely to contribute significantly to the increased serum enzyme levels in this disease.

It was not possible, using this technique, to determine whether the maturation arrest which also affects the myeloid series also results in an increased enzyme content in these cells. The degree of enzyme inhibition using cold ethanol and acetone fixation appears to be sufficient to give virtually negative formazan production in late myeloid cells.

In both normoblastic and megaloblastic erythropoiesis there is a progressive decrease in intra- and extra-mitochondrial enzyme activity with increasing maturation. This was also noted by Hayhoe (1963) in developing normoblasts. Neither the normoblastic hyperplasia of secondary polycythaemia and haemolytic anaemia, nor the maturation arrest of megaloblastosis significantly alters this. There was also no disproportionate reduction in the activity of α -GPD-NAD in megaloblasts, or in normoblasts undergoing hyperplasia, and the results for these examples of benign bone marrow hyperplasia contrast with reports of a selective decrease in α -GPD-NAD activity in mouse leukaemic cells (Sacktor and Dick, 1960).

**PART III: HYDROGEN TRANSPORT SYSTEMS IN ERYTHROID
CELLS IN CHRONIC RENAL FAILURE**

1. Introduction

The normal survival and biconcave shape of the mature erythrocyte is dependent on the effectiveness of its membrane ionic pump. This pump regulates Na^+ and K^+ transport across the cell membrane and derives its energy from glycolysis (Harris, 1963). The mature red cell no longer possesses nuclear DNA or cytoplasmic RNA and cannot synthesise new protein. Thus it is dependent on its inherited enzyme content and, as the activity of this declines with age, the membrane pump gradually becomes less effective until osmotic swelling and disruption of the cell occurs as a terminal event.

In the event of an inherited enzyme deficiency in the glycolytic pathway (e.g. pyruvate kinase deficiency) then glycolysis and ATP synthesis is impaired, the membrane pump becomes inoperative prematurely, and a shortened red cell survival results. A similar reduction in red cell survival might also occur in the presence of a glycolytic enzyme inhibitor since the erythrocyte, without an effective tricarboxylic acid cycle respiratory pathway, is dependent on glycolysis for ATP synthesis.

Morgan, Morgan and Thomas (1963) and Emerson et al. (1965) reported that the glycolytic enzyme LD, and the enzyme corresponding to its fastest migrating isoenzyme - hydroxybutyrate/

hydroxybutyrate dehydrogenase (HBD), were inhibited by ultra-filtrates of blood from uraemic patients. Morgan and Morgan (1964) also reported an inhibitory effect of such ultra-filtrates on the glucose utilisation of normal red cells. Thus it is theoretically possible that LD inhibition might impair glycolysis and therefore red cell survival in chronic renal failure; in support of this hypothesis, Rees, Scheitlin, Pond, McManus, Guild and Merrill (1957) showed that the erythrocytes in uraemia have a diminished reserve of high-energy phosphate. Patients with chronic renal failure maintained on regular haemodialysis show decreased transfusion requirements (Comty, Baillo and Shaldon, 1965; Shaldon, 1968) which is also in favour of a plasma factor adversely affecting either red cell survival and/or erythroid cell production by the marrow.

It is also theoretically possible that the marrow hypoplasia associated with high urea levels could result from inhibition of glycolysis since mitotic division is believed to have a requirement for glycolysis (Bullough, 1952; 1954), and since many biosynthetic processes within the cell are also dependent on glycolysis and/or respiration.

2. Methods

Representative enzymes from the Embden-Meyerhof pathway (LD), tricarboxylic acid cycle (MD) and pentose phosphate shunt (GGPD) were studied cytochemically in marrow erythroblasts using the cold ethanol fixation technique with PVA added to the incubation solution. Since this cytochemical technique requires a fixative, which results in some enzyme inhibition, the method is theoretically not ideal for the demonstration of an enzyme inhibitor. While it is unlikely that the fixative would have a greater enzyme inhibitory effect on uraemic, compared with normal, erythroblasts, the inevitable small degree of enzyme inhibition due to fixation might, however, be sufficient to mask the effect of a weak plasma inhibitor.

A parallel study was therefore carried out on the mature circulating red cells from the same patients in order to determine, spectrophotometrically, their LD activity. Venous blood was collected into acid citrate/dextrose and haemolysates prepared within 60 min by washing three times in 0.85% sodium chloride, then diluting 1/20 in distilled water and freezing and thawing. The supernatants, obtained after centrifugation for 10 min at 2,000 g at 4°C, were diluted 1/10 in 0.067M phosphate buffer/

buffer, pH 7.4, and their enzyme activity measured spectrophotometrically at 25°C by the method of Wróblewski and La Due (1955). Emerson et al. (1965) also reported inhibition of HBD in chronic renal failure and this enzyme was therefore measured spectrophotometrically in the same haemolysates by the method of Rosalki and Wilkinson (1960). HBD corresponds in activity to the anodic isoenzymes of LD (Rosalki and Wilkinson, 1960) and these isoenzymes are relatively resistant to heat inactivation (Plagemann, Gregory and Wróblewski, 1961; Latner and Skillen, 1963). The marrow cytochemical technique for the demonstration of LD activity was shown to demonstrate predominantly the anodic isoenzymes, equivalent to HBD, by processing duplicate marrow smears for LD with and without prior incubation of the smear at 60°C for 60 min. There was no detectable decrease in formazan deposition in the heat inactivated smears. Thus both the marrow cytochemical technique and the red cell haemolysate spectrophotometric technique demonstrated the fastest migrating component of LD, which is the fraction inhibited by uraemic blood ultra-filtrates (Emerson et al., 1965); it was anticipated that the combination of the two techniques would be sufficiently sensitive to detect significant erythroid cell enzyme inhibition.

3. Patients Studied

Eighteen patients with chronic renal failure and in a relatively steady state were studied (see Tables IX and X). It is difficult to achieve steady state conditions in chronic renal failure but in this group of patients there was no overt blood loss and only four patients had been transfused within the preceding 90 days. All patients had serum vitamin B₁₂ levels greater than 270 pg/ml (normal range 140-1,000 pg/ml), while four patients had serum folate levels below 3.5 ng/ml. These patients were, however, taking oral broad-spectrum antibiotics at the time of assay. Erythropoiesis was normoblastic in maturation in all 10 patients in whom bone marrow studies were performed although a few giant metamyelocytes were present in five cases.

The mean level for creatinine clearance for the group was 2.7 mg/min and for urea 274 mg/100 ml. Five patients had received a previous solitary peritoneal dialysis and two patients were maintained on chronic intermittent haemodialysis. In these patients the marrow and blood specimens were taken immediately prior to the next dialysis.

TABLE IX

Clinical and Biochemical Data for 18 Patients
With Chronic Renal Failure

CASE NO.	AGE (YR)	SEX	RENAL DISEASE	BLOOD-UREA (mg/100 ml)	SERUM-CREAT-ININE (mg/100 ml)	CREAT-ININE CLEAR-ANCE (ml/min)
1	19	F	Glomerulonephritis	183	17.4	0.6
2	48	F	Polycystic kidneys	234	6.6	4.5
3	17	F	Glomerulonephritis	384	19.8	1.2
4	43	F	Polycystic kidneys	230	12.2	0
5	41	M	Uric-acid nephropathy	87	4.6	7.0
6	52	M	Hydronephrosis	231	16.2	2.6
7	53	F	Unknown	300	20.4	0.8
8	53	M	Polycystic kidneys	291	15.0	2.5
9	47	M	Polycystic kidneys	168	11.4	-
10	48	M	Unknown	390	15.0	5.8
11	49	M	Unknown	417	19.8	0
12	47	F	Unknown	366	14.0	1.6
13	64	M	Myelomatosis tubulopathy	237	25.8	1.0
14	22	M	Pyelonephritis	423	18.3	2.3
15	22	M	Glomerulonephritis	288	5.1	10.0
16	32	M	Pyelonephritis	182	16.5	4.0
17	20	F	Pyelonephritis	291	18.0	0
18	37	F	Pyelonephritis	234	16.8	1.8

TABLE XHaematological Data for 18 Patients With
Chronic Renal Failure

CASE NO.	Hb (g/100 ml)	SERUM-FOLATE (ng/ml)	ERYTHRO-BLASTS IN MARROW (%)	METAMYELO-CYTES IN MARROW (% GIANT)	DAYS SINCE LAST TRANSFUSION (UNITS OF BLOOD GIVEN)
1	6.4	3.8	8.4	7	..
2	9.6	7.2	7.2	12	14 (5)
3	6.5	4.9	18.6	0	..
4	4.1	> 21.0	4.0	0	9 (1)
5	6.9	> 21.0	16.0	8	..
6	7.4	3.0	22.4	3	18 (1)
7	8.5	0.6	8.0	3	..
8	9.0	7.0	29.4	0	..
9	11.5	4.6	25.8	0	..
10	7.3	11.7	26.6	0	..
11	6.6	2.6
12	11.6	5.8
13	7.2	5.0	10 (2)
14	6.3	7.2
15	7.4	9.2
16	7.2	4.0
17	5.1	> 21.0
18	6.3	2.2

4. Results

4.1 Bone marrow cytochemical study At least 10, and usually 25, erythroblasts were scored at each of the three stages of maturation for each enzyme (see Table XI). The mean scores were compared with the mean scores obtained for 10 normal control patients who were neither anaemic nor uraemic. The mean percentage of erythroblasts was similar in the two groups - 16.6% and 15.5% respectively. Comparison between the mean enzyme scores of the two groups showed no significant ($P > 0.1$) difference for any enzyme at any of the three stages of maturation. The mean enzyme score for the patients who showed a small number of giant metamyelocytes in the marrow film was no higher than the mean score for the remainder.

4.2 Red cell haemolysate study The enzyme activity of red cell haemolysates from patients 3-18 were studied and the mean enzyme levels for LD and HBD compared with the levels obtained from 10 non-uraemic control patients matched for age and sex (see Table XI). The mean levels for LD and HBD were not significantly ($P > 0.1$) lower in the 16 uraemic patients, and there was again no increase in the mean enzyme level for those uraemic patients with reduced serum folate activity.

5./



TABLE XI

Enzyme Activity in Marrow Erythroblasts and Blood Erythrocytes

	MARROW ERYTHROBLASTS (ARBITRARY SCORE, MEAN \pm SD)									RED-CELL HAEMOLYSATE (IU/ml)	
	EARLY			INTERMEDIATE			LATE				
	LD	MD	G6PD	LD	MD	G6PD	LD	MD	G6PD	LD	HBD
Uraemic patients	586 \pm (-46)	395 \pm (-90)	416 \pm (-73)	464 \pm (-78)	338 \pm (-129)	308 \pm (-79)	378 \pm (-123)	295 \pm (-63)	169 \pm (-58)	25.8 \pm (-3.8)	18.9 \pm (-2.3)
Matched controls	574 \pm (-67)	434 \pm (-135)	472 \pm (-97)	443 \pm (-102)	301 \pm (-117)	339 \pm (-35)	373 \pm (-92)	256 \pm (-73)	184 \pm (-62)	23.8 \pm (-3.4)	19.1 \pm (-1.8)

5. Discussion

It is widely accepted that the metabolites which accumulate in chronic renal failure can adversely influence the metabolic function of marrow and blood cells: platelet function (Castaldi, Rozenberg and Stewart, 1966); lymphocyte transformation with PHA (Elves, Israëls and Collinge, 1966); erythroblast maturation and proliferation in vitro (Sacchetti, 1953; Markson and Rennie, 1956; Berman and Powsner, 1959); reticulocyte maturation (Baldini and Pannacciulli, 1960); and red cell autohaemolysis (Giovannetti, Balestri and Cioni, 1965) have all been shown to become abnormal in the presence of a high urea level and to be corrected, at least partially, by contact with non-uraemic plasma in vitro or by dialysis. Thus it is possible that an enzyme inhibitor in uraemic plasma could also adversely affect enzyme activity within erythroid cells.

Hampers, Streiff, Nathan, Snyder and Merrill (1967) have described megaloblastic erythropoiesis due to folate deficiency in patients maintained on dialysis, and since megaloblasts and macrocytes contain an increased activity of LD and HBD it was necessary to exclude megaloblastic change in the uraemic patients. The presence of megaloblasts in this group of patients would result/

result in a false high enzyme level which might mask the effect of an inhibitor. The four patients with serum folate levels below 3.5 ng/ml (see Table X) were receiving antibiotics at the time of assay and inhibition of the growth of the test lactobacillus at high serum concentrations in the micro-biological assay procedure in two of these patients indicated an antibiotic inhibitory effect. There was no corresponding megaloblastic change in the marrow of two of these patients (no marrow examination was performed in the remaining two) and in the other patients in whom the marrow was examined the presence of a few giant metamyelocytes did not increase the enzyme score. This degree of megaloblastoid change would not be sufficient to increase demonstrable LD, MD and G6PD activity, as shown previously (see II.3).

The results imply that the LD inhibitor in uraemic plasma does not significantly depress the in vivo activity of this enzyme in erythroid cells, either in the bone marrow or blood. This form of enzyme inhibition is unlikely, therefore, to be the mechanism which results in the bone marrow production defect and the decreased red cell survival of chronic renal failure.

**PART IV: HYDROGEN TRANSPORT SYSTEMS
IN LEUCOCYTES**

1. Introduction

Magill, Wróblewski and La Due (1959) reported elevated serum LD levels in 77% of 30 adults and 70% of 10 children with acute leukaemia, and Blanchaer, Green, MacLean and Hollenberg (1958) also reported elevated levels in 87% of 16 cases of acute leukaemia. In carcinoma, in general, approximately 50% of cases show raised serum levels of LD (Wilkinson, 1962).

In addition to these reports of increased enzyme levels, there have been reports of increased lactic acid levels in acute leukaemia of a degree sufficient to result in systemic lactic acidosis (Field, Block, Levin and Rall, 1966; Rothe and Porte, 1970). In the seven cases described by Field et al. (1966) the lactic acidosis developed despite apparently adequate tissue oxygenation and it subsequently subsided as the patients achieved haematological remission. There was a poor correlation between the serum LD level and the blood white cell count and this implied an additional source of LD other than the disintegration of blood leucocytes.

These reports of elevated serum LD activity are analagous to the findings in megaloblastic anaemia (see II.1) and since leukaemic blast cells also show a maturation arrest in the mitotic cycle it is possible that the/

the death of marrow blast cells contributes significantly to the raised serum levels in leukaemia. This in itself would justify a cytochemical study of LD activity in leukaemic blast cells, but there is evidence for a much more fundamental alteration in the activity of glycolytic enzymes in solid tumours and leukaemia which may be related to neoplastic change and which warrants a more detailed examination of glycolytic and respiratory enzyme activity.

2. Aerobic Glycolysis and the Warburg Hypothesis

Tumour cells contain similar enzymes to those of normal tissues; no new or abnormal enzymes have been found although certain enzymes may be absent, or significantly reduced in activity, compared with the tissue of origin of the tumour. The enzyme systems which are normally retained within malignant cells include the basal systems required for energy production to allow growth and reproduction, rather than those enzymes concerned solely with the specialised functions of differentiated cells. Thus an abnormality of the former is more likely to represent a direct consequence of neoplastic change rather than merely dedifferentiation.

Warburg (1930, 1956) first suggested that neoplastic cells/

cells show a characteristic abnormality of the basic energy generating systems of respiration and glycolysis. He observed that tumour cells almost always show a higher rate of aerobic glycolysis than normal cells - the ratio of aerobic glycolysis to respiration was 3-4:1 for tumour cells and approximately 1:1 for normal cells - and most workers now accept that there is a fundamental alteration in glycolysis in neoplasia (Woods, Sanford, Burke and Earle, 1959; Ashmore, Weber, Banerjee and Love, 1961; Broadfoot, Walker, Paul, Macpherson and Stoker, 1964; Paul, 1966).

Warburg also argued, however, that this abnormality arose because neoplastic cells, in the course of their malignant transformation, suffered permanent damage to the mitochondrial electron transport system. This resulted in a preferential increase in glycolysis even under aerobic conditions and favoured autonomous growth under conditions of lowered oxygen tension. It was a corollary of this hypothesis that neoplastic cells should have a lower respiratory rate than normal cells and that the Pasteur effect was limited (i.e. the high rate of anaerobic glycolysis decreased only marginally when oxygen was introduced).

This part of his hypothesis has never been accepted, largely/

largely because normal cells also show a decrease in respiration and an increase in glycolysis when deprived of oxygen in vitro (Paul and Pearson, 1957; Warburg, Gawehn, Geissler, Schröder, Gewitz and Volker, 1958). Neoplastic cells in a solid tumour in vivo are also probably exposed to lower than normal oxygen tensions and thus the Warburg hypothesis can be explained on the basis of an inadequate blood supply to solid tumours. It has also been widely recognised that the oxygen consumption of experimental tumours is not diminished under aerobic conditions and that electrons can reach oxygen almost as readily as in normal cells (Weinhouse, 1956); thus the concept of an irreversible defect in the electron transport chain has not been substantiated. Many factors such as oxygen tension, cell density and pH have been shown to influence glycolysis and respiration in vitro and these observations provide an explanation for many of the early experimental studies which indicated preferential aerobic glycolysis in malignant cells.

Even when these factors are taken into consideration, the more recent work of Woods et al. (1959); Ashmore et al. (1961); and Broadfoot et al. (1964) suggest that an/

an increase in aerobic glycolysis, but in the presence of co-existing normal respiration, remains a characteristic of malignant cells. Under controlled conditions, with exactly the same oxygen uptake rates, Broadfoot et al. (1964) transformed BHK 21 baby hamster kidney cells with SE polyoma virus and obtained a two-fold increase in glucose utilisation, and also in lactate production (Paul, 1966).

A fundamental study of the rate controlling factors in glycolysis and respiration would therefore seem important in any attempt to explain this preferential aerobic glycolysis.

3. Integration of Mitochondrial Respiration and Extra-mitochondrial Glycolysis

The three major metabolic pathways - the Embden-Meyerhof pathway, tricarboxylic acid cycle and pentose phosphate shunt (see Fig. 2) - operate in an equilibrium which appears to depend on competition for certain metabolites common to each of them. This competition is the probable basis for the Pasteur and Crabtree effects:

Pasteur effect - the inhibition of glycolysis which occurs in facultative anaerobes in the presence of oxygen. Conversely, in an atmosphere of nitrogen there is an increase/

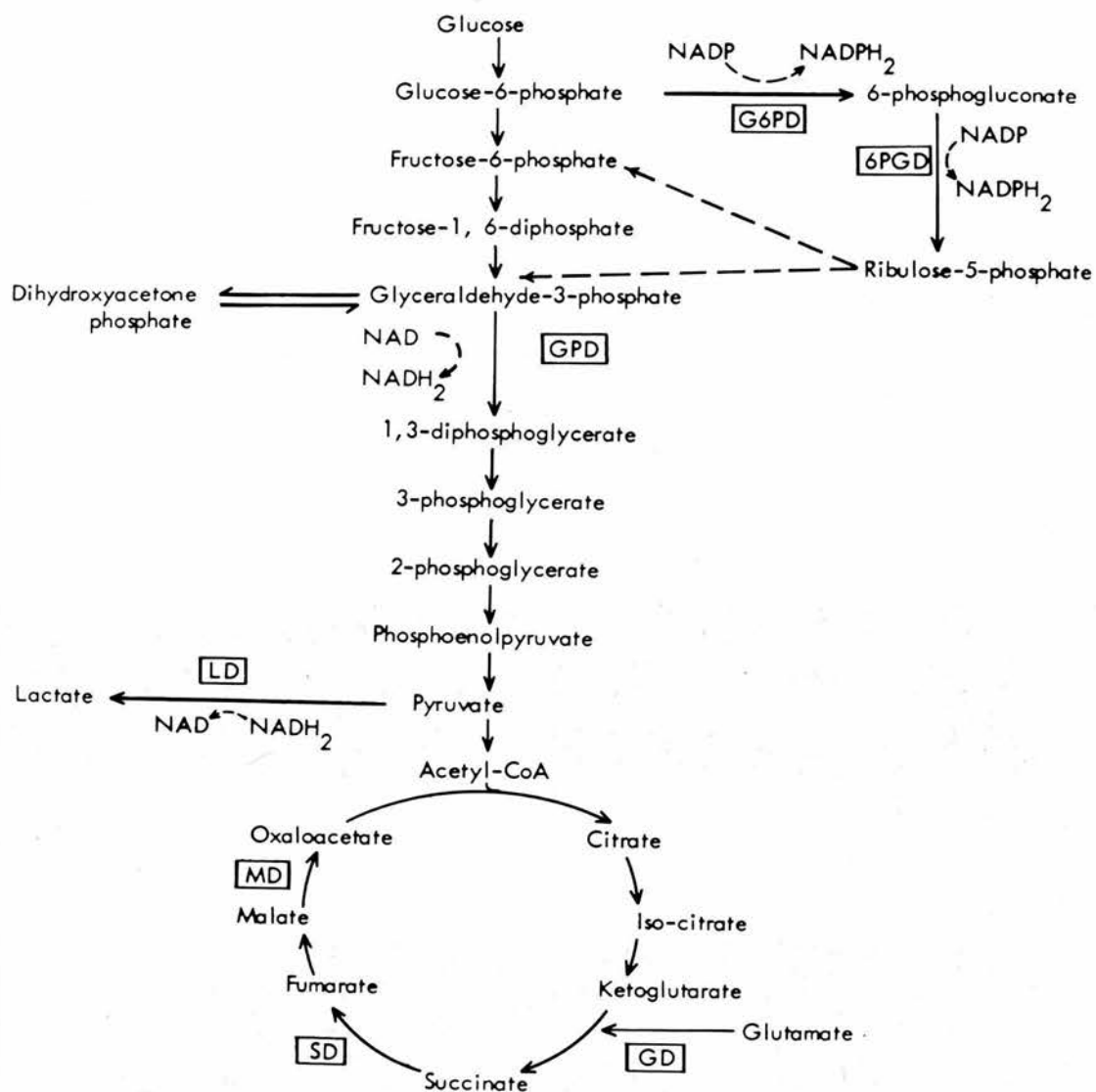


Fig. 2. Diagram of Embden-Meyerhof pathway, pentose phosphate shunt and Krebs' tricarboxylic acid cycle. See 'Abbreviations Used in the Text' for key to enzymes.

increase in glucose consumption and lactate production.

Crabtree ('inverse Pasteur') effect - the depression of respiration which results from an increase in glucose concentration.

The metabolites involved in the balance between glycolysis and respiration include the concentration of inorganic phosphate and the ratios of ADP:ATP and NAD:NADH₂ (Lehninger, 1965). Lynen (1958) suggested that since high concentrations of inorganic phosphate are required to achieve maximal rates of glycolysis, whereas oxidative phosphorylation may proceed at maximal rates even at low phosphate concentrations, then the inhibition of glycolysis exerted by respiration (Pasteur effect) is due to the relatively low levels of intracellular phosphate resulting from oxidative phosphorylation.

Ratner and Racker (1950) and Chance, Garfinkel, Higgins and Hess (1960) suggested that it was the ratio of the concentrations of ADP and ATP which formed the basis for integration of glycolysis and respiration; Baierlein and Foster (1968) extended this hypothesis to show that in isolated leucocytes the concentration of phosphofructokinase is largely responsible for the Pasteur effect in leucocytes. Phosphofructokinase is competitively inhibited by ATP, and/

and reactivated by ADP and inorganic phosphate. Thus the transition to aerobic conditions, with a resulting increase in ATP, and decrease in ADP and phosphate concentrations, inhibits phosphofructokinase activity and therefore glycolysis. The role of oxygen is therefore an indirect one and the Pasteur effect is largely due to the ATP formed during respiration. Conversely, if the ATP concentration falls below a certain level it is subsequently restored by increased activity of phosphofructokinase.

Boxer and Devlin (1961) provided substantial evidence for a control mechanism based on the relative concentrations of the co-enzyme NAD and its reduced form NADH_2 . In the Embden-Meyerhof pathway there is a single oxidative reaction - the oxidation of glyceraldehyde-3-phosphate to 1,3-diphospho-glyceric acid (see Fig. 2). NAD is the essential cofactor for this reaction and, since it is present only in catalytic amounts in mammalian cells (Kaplan, 1960), an efficient method for its reoxidation from the reduced form NADH_2 is clearly essential for the maintenance of glycolysis. Under aerobic conditions the hydrogen ions from NADH_2 pass via an α -glycerolphosphate shuttle system (see Fig. 3) to be oxidised via the intra-mitochondrial respiratory chain. Under/

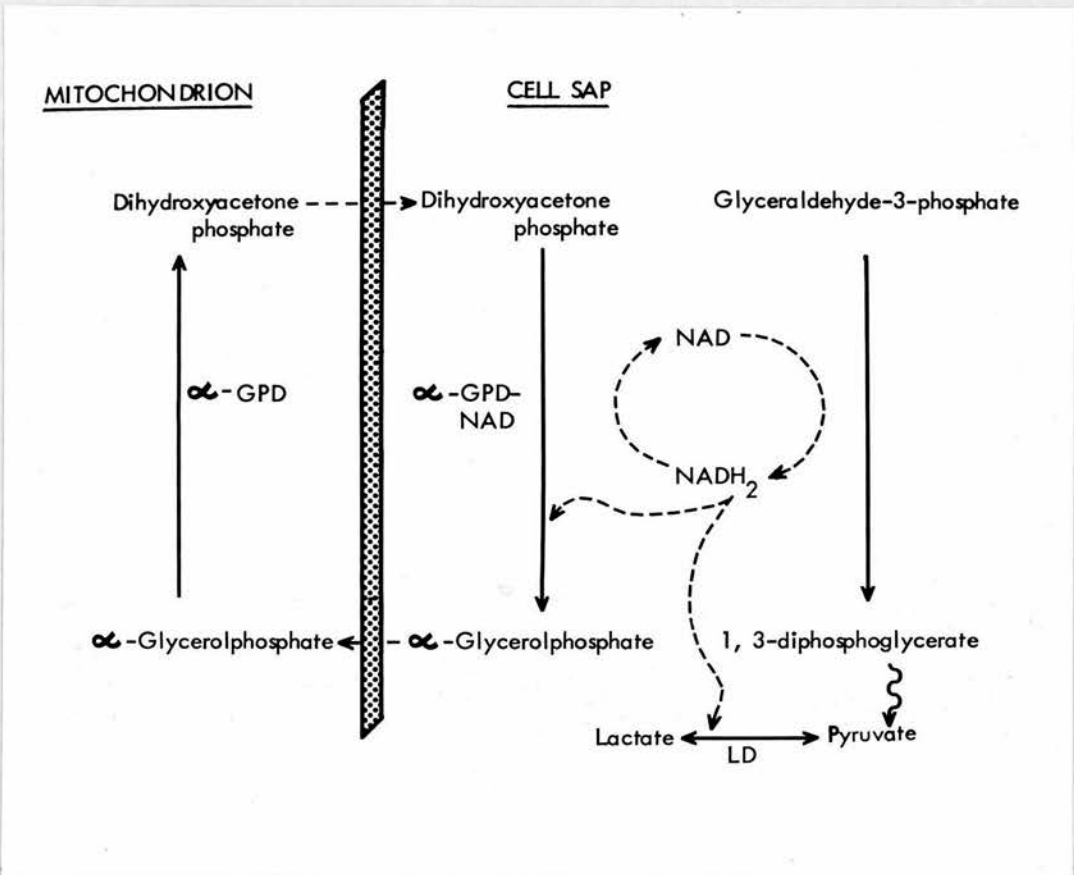


Fig. 3. Diagram of α -glycerolphosphate shuttle system.

Under anaerobic conditions the α -glycerolphosphate shuttle system is not used and the hydrogen ions are instead taken up in the reduction of pyruvate to lactate, catalysed by LD. Thus there is a resulting increase in LD activity and in lactate production.

Each of these theories is based on the principle of competition between the extra-mitochondrial glycolytic pathway and the intra-mitochondrial respiratory chain for a metabolite required by each of them - inorganic phosphate; ADP:ATP; and NAD:NADH₂. When the demand increases in one pathway then the resulting decrease in availability affects the other pathway; for example, the inhibitory effect of high glucose concentrations on respiration (Crabtree effect) is probably due to the glucose causing increased competition by the glycolytic pathway for inorganic phosphate and pyridine nucleotides, thus providing less for oxidative phosphorylation (Staunton West, Todd, Mason and Van Bruggen, 1966).

Thus, inter-relationships between extra-mitochondrial glycolysis and intra-mitochondrial respiration are of profound importance in a study of the preferential aerobic glycolysis of tumour cells, and the shuttle systems operating between them across the mitochondrial membrane become of great significance.

4. Mitochondrial Membrane Shuttle Systems

The enzymes and cofactors involved in the Embden-Meyerhof pathway have been studied both by cell fractionation procedures (Kennedy and Lehninger, 1949; Wu and Racker, 1959) and in intact cells (Lynen, 1958; Chance and Hess, 1959) and shown to be primarily located in the cell sap of the cytoplasm. Thus the mitochondrial membrane forms a physical barrier between the glycolytic pathway and the intra-mitochondrial electron transport chain. The permeability of this barrier to substrate and cofactors may therefore play a major role in regulating the balance between glycolysis and respiration (Lynen, 1958; Chance and Hess, 1959).

Under aerobic conditions the hydrogen ions of the NADH_2 generated in the oxidation of glyceraldehyde-3-phosphate to 1,3-diphosphoglyceric acid are oxidized by the intra-mitochondrial electron transport chain which leads to molecular oxygen. Lehninger (1951), however, demonstrated that the intact mitochondrial membrane is impermeable to NADH_2 itself and that it becomes permeable only when pathologically damaged by osmotic imbalance, uncoupling agents or by chemical or mechanical means. In the intact cell this impermeability is overcome by one, or a number of, shuttle systems which permit the transfer of/

of hydrogen from the cell sap into the mitochondria.

4.1 α -Glycerolphosphate shuttle The most fully understood, and probably the most important, of these shuttle systems is the α -glycerolphosphate-dihydroxyacetone phosphate system described by Bucher and Klingenberg (1958) and Sacktor (1958). The interaction of aldolase and fructose-1,6-diphosphate results in the production of equimolar amounts of dihydroxyacetone phosphate and glyceraldehyde-3-phosphate (see Fig. 2). Triosephosphate isomerase establishes an equilibrium between the two products which is in favour, by 20:1, of dihydroxyacetone phosphate.

In the presence of the extra-mitochondrial enzyme α -GPD-NAD, dihydroxyacetone phosphate can accept hydrogen from NADH_2 to form L- α -glycerolphosphate, which is then able to pass freely into the mitochondrion while the NADH_2 is reoxidised to NAD (see Fig. 3). Within the mitochondrion, α -glycerolphosphate becomes the substrate for an insoluble, NAD-independent enzyme α -GPD (Green, 1936) and is oxidised to dihydroxyacetone phosphate. The hydrogen released by this oxidation follows the respiratory chain to molecular oxygen while the dihydroxyacetone phosphate probably diffuses out into the cell sap/

sap to complete the cycle (Boxer and Devlin, 1961) .

This shuttle is the major route for NADH_2 oxidation in insect musculature, which has low LD activity (Estabrook and Sacktor, 1958), but it also functions in mammalian brain and other tissues (Sacktor, Packer and Estabrook, 1959; Klingenberg and Slenczka, 1959) . α -glycerolphosphate is present in considerable concentration in most normal human tissues even under aerobic conditions (Leva and Rapoport, 1943) . . There is evidence that the shuttle is also of importance in haemopoietic tissues and is probably the major route for NADH_2 oxidation under aerobic conditions. Wagner et al. (1956) showed that the endogenous oxygen consumption of leucocyte suspensions could be reduced almost to zero by dialysing out endogenous substrate and could be restored by adding α -glycerolphosphate as substrate. Wagner et al. (1956) also provided evidence that α -GPD is the most active respiratory enzyme in leucocyte-platelet suspensions and concluded that the endogenous respiration of leucocytes is largely dependent on α -glycerolphosphate as essential substrate. The presence of α -GPD-NAD has been demonstrated biochemically in normal lymphoid tissue (Sacktor and Dick, 1960), histochemically in lymphocytes and macrophages (Wulff, 1963) and cytochemically in lymphocytes/

lymphocytes, polymorphs and monocytes (Gough and Elves, 1967) .

4.2 α -Glycerolphosphate shuttle in malignant tissues Under anaerobic conditions, NADH_2 is not reoxidised via the α -glycerolphosphate shuttle and the hydrogen is instead taken up in the reduction of pyruvate to lactate (see Fig. 3) . This would also occur under aerobic conditions if the mitochondria were scanty in number, if the respiratory pathway was damaged or blocked, or if the α -glycerolphosphate shuttle enzymes were deficient. Holzer, Glogner and Sedylmayr (1958) first demonstrated decreased extra-mitochondrial α -GPD-NAD activity in malignant tissue in a study of Ehrlich ascites and Yoshida hepatoma cells, and similar results were subsequently obtained in a variety of virus- and carcinogen-induced transplanted tumours in rats (Delbrück, Schimassek, Bartsch and Bücher, 1959; Boxer and Shonk, 1960) . The mitochondrial counterpart of this enzyme, α -GPD, has usually been reported to be normal or increased in activity in malignant compared with normal tissues (Boxer, 1965) . Boxer and Shonk (1960) showed that the reduction in activity of α -GPD-NAD was not due to the presence of an inhibitor since the addition of tumour extracts to normal tissue homogenates did not inhibit enzyme activity.

A reduction in activity of α -GPD-NAD has also been shown, by tissue homogenate studies, in a variety of human tumours (Boxer, 1965) and this reduction is probably not a reflection of rapid growth per se since an increase in activity is seen during periods of rapid growth in non-malignant regenerating liver cells (Shonk, Morris and Boxer, 1965).

When the activity of α -GPD-NAD and of the α -glycerol-phosphate shuttle is reduced the compensatory uptake of hydrogen by the pyruvate-lactate reaction results in increased activity of LD. Boxer (1965) found the ratio of LD: α -GPD-NAD to be increased six-fold in homogenates of neoplastic, compared with normal, tissue. A similar enzyme pattern has been described in malignant haemopoietic tissue. Bücher and Klingenberg (1958) reported high LD and low α -GPD-NAD activity in lymphosarcoma, and Sacktor and Dick (1960) found a 10-fold increase in LD activity and an 85% reduction in α -GPD-NAD activity in B82T leukaemia in C58 mice: the ratio of LD: α -GPD-NAD reached 100:1, compared with 10:1 in normal mouse lymphoid tissue homogenates. A similar abnormality was found in L1210 mouse leukaemia (Sacktor and Dick, 1960). In preliminary studies Sacktor (1968) found an identical pattern in leucocyte homogenates from patients with acute and chronic lymphatic leukaemia.

A number of exceptions to the low α -GPD-NAD activity in animal tumour cells have been reported. Morris, Sidransky, Wagner and Dyer (1960) found normal activity in the ascitic Ehrlich-Lettre tumour of the mouse and Shonk, Morris and Boxer (1965) observed that the LD: α -GPD-NAD ratio was higher in rapidly growing, compared with slowly growing, hepatomas. Diengdoh and Chayen (1969) also showed an increase in both LD and α -GPD-NAD in benzpyrene induced skin tumours in the mouse.

4.3 Acetoacetate- β -hydroxybutyrate shuttle An alternative shuttle system, analagous to the α -glycerolphosphate shuttle, but involving acetoacetate and β -hydroxybutyrate, has been described by Devlin and Bedell (1960). In this pathway, extra-mitochondrial NADH_2 reduces acetoacetate to β -hydroxybutyrate and the catalytic action of the enzyme β -HBD, sited on the mitochondrial membrane, results in the subsequent intra-mitochondrial oxidation of β -hydroxybutyrate to acetoacetate (see Fig. 4). β -HBD is believed to act at two loci on the mitochondrial membrane - at one of these it can react with external β -hydroxybutyrate and NAD, and at the other it can transfer hydrogen to the internal electron transport chain (Lehninger, Sudduth and Wise, 1960; Devlin and Bedell, 1960/

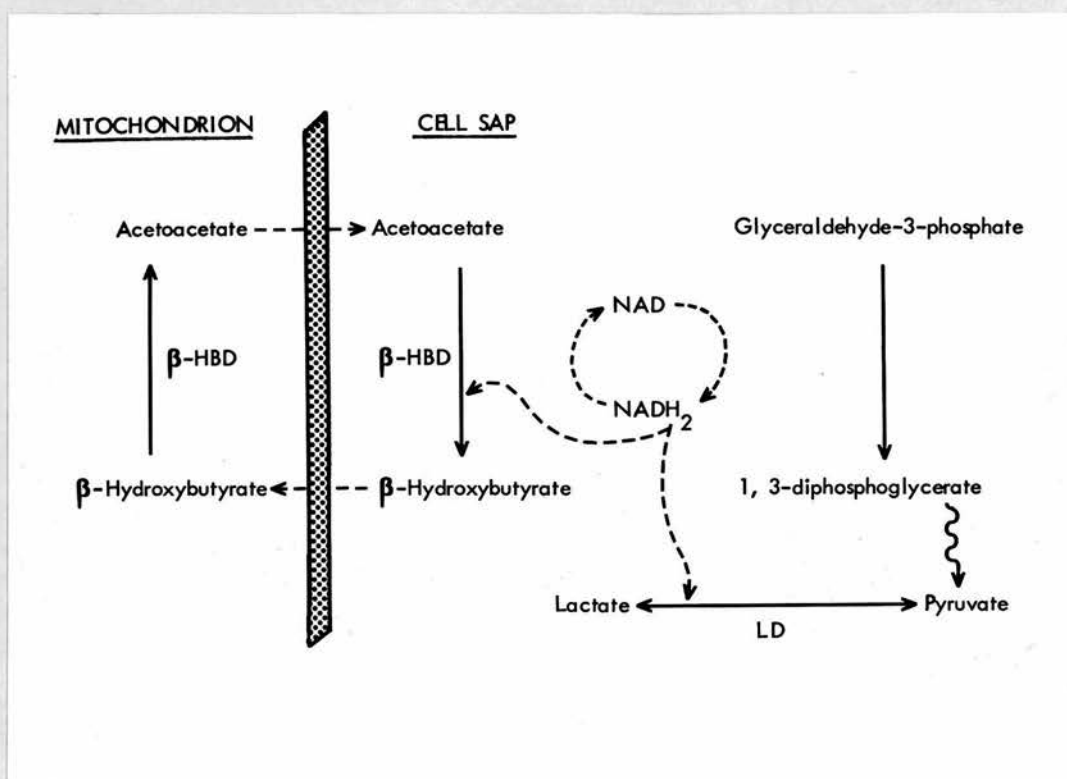


Fig. 4. Diagram of acetoacetate-β-hydroxybutyrate shuttle system.

1960; Krebs, Eggleston and D'Alessandro, 1961).

The absence of either, or both, the external or internal form of β -HBD has been described in certain tumours (Boxer and Devlin, 1961).

4.4 Malate dehydrogenase shuttle A similar shuttle system to that described for the soluble and mitochondrial forms of α -GPD has been suggested for the enzyme MD (Kaplan, 1960). MD is widely distributed throughout the mitochondrial, microsomal and supernatant fractions of homogenates and catalyses the reversible conversion of L-malate into oxaloacetate (Wilkinson, 1962).

Delbrück, Schimassek, Bartsch and Bücher (1959) and Thorne (1960) described two forms of MD - one intra- and one extra-mitochondrial. Kaplan and Ciotti (1961), Siegel and England (1962) and Roodyn, Suttie and Work (1962) subsequently confirmed that the two forms of MD were different proteins. Kaplan (1960) envisaged that the oxidation of NADH_2 in the cell sap by the conversion of oxaloacetate to malate resulted in the entry of malate into the mitochondrion (see Fig. 5), where it subsequently became oxidised by the mitochondrial-bound form of MD. The resulting oxaloacetate thereafter re-entered the cell sap to complete the cycle.

4.5/

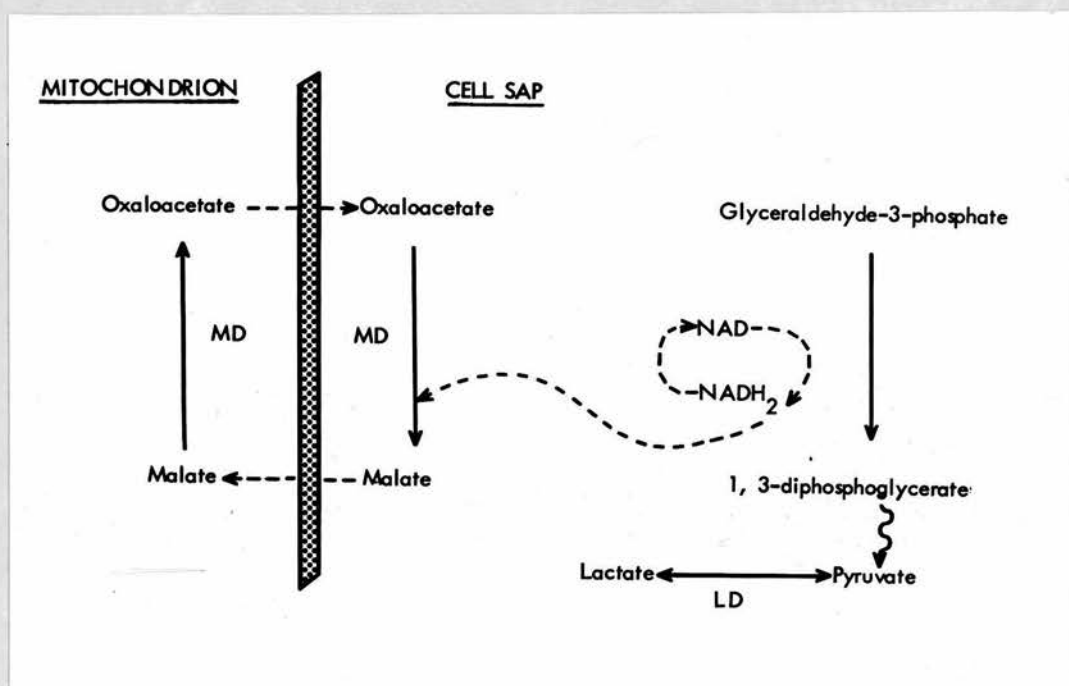


Fig. 5. Diagram of malate dehydrogenase shuttle system.

4.5 NAD-NAD Transhydrogenase and other shuttle systems The evidence for the existence of other shuttle systems is less convincing. Kaplan, Colowick and Neufeld (1953) and Kaplan, Colowick, Zatman and Ciotti (1953) suggested that the extra-mitochondrial hydrogen of NADH_2 could be transferred to the intra-mitochondrial electron transport system by means of a specific NAD-NAD transhydrogenase, but this enzyme has subsequently been shown to have an entirely intra-mitochondrial function (Stein, Kaplan and Ciotti, 1959).

While there is convincing evidence for the existence of this type of shuttle system, which may contribute to the regulation of the Pasteur and Crabtree effects, the relative importance of individual shuttle systems has yet to be determined for specific normal tissues, and it is unknown whether all such systems operate simultaneously, and can substitute for one another if necessary, or whether a given tissue is dependent on only one pathway. It is likely, however that the α -glycerolphosphate shuttle is the major system in haemopoietic cells.

There are difficulties, moreover, in assessing the significance of an apparent loss of enzyme activity in some of these pathways in neoplastic tissue : apparent reduction/

reduction in enzyme activity in a homogenate may merely represent a failure to obtain a valid comparison with an equivalent normal tissue; some enzyme differences may be due to the effects of hypoxia or variation in cell proliferative rate; and reduction in enzyme activity may simply represent loss of specialist function in a tissue and may correspond more to cell immaturity rather than neoplastic change per se. Many of these difficulties are inherent to tissue homogenate studies and it would seem, therefore, that a cytochemical study of the enzymes involved in these pathways in leukaemic blast cells, and in developing normal marrow cells, would allow an interpretation of the activity of these pathways in relation to cellular differentiation and neoplastic change.

5. Development of Method

5.1 Disadvantages of fixation Both cold ethanol and 60% acetone result in dehydrogenase enzyme inhibition. Cold ethanol fixation was found to inhibit enzyme activity in mature myeloid cells (see II.4); Quaglino and Hayhoe (1960) and Hayhoe et al. (1964) also reported little or no formazan production for SD and LD beyond the metamyelocyte stage of differentiation in acetone-fixed/

acetone-fixed myeloid cells. This inhibitory action would therefore prevent a study of enzyme activity in relation to stage of maturation in late myeloid cells and would also raise difficulties in interpretation if decreased activity of α -GPD-NAD were found in leukaemic cells.

The lipid-clearing action of acetone and ethanol might also influence hydrogen transport across the mitochondrial membrane. The principal mitochondrial enzymes concerned with respiration and phosphorylation are believed to be either embedded in, or closely associated with, the mitochondrial membrane (Bourne, 1964). Mitochondria consist of 30-40% by weight of lipid, of which 90% is phospholipid, and most of this is concentrated in the membrane. Green and Fleischer (1963) have stressed the fundamental importance of phospholipids in maintaining the integrity of the electron transport and oxidative phosphorylation systems. Lipid arrays probably play an important role in maintaining the correct spatial relationships of enzyme systems in mitochondria and any agent which disturbs their inter-relationships may interfere with membrane shuttle systems. Fleischer, Brierley, Klouwen and Slautterback (1962) have demonstrated that when intact mitochondria are treated with aqueous acetone/

acetone this results in removal of 80% of the total phospholipid and thus a fixative or any surface- or lipophilic-agent is likely to act as a respiratory uncoupler (Roodyn, 1967) .

Walder (1970) and Walter (1970) have recently provided electromicrograph evidence that mitochondrial membranes are also damaged by freezing and thus both the 60% acetone and the cold ethanol fixation techniques would not seem ideally suited for a detailed examination of membrane shuttle systems in leukaemic cells, if only in respect of difficulties in interpretation if a decrease in enzyme activity were found. An attempt was therefore made to develop a cytochemical technique which did not require cell fixation in order to achieve adequate preservation.

5.2 Investigation of supravital techniques The cold ethanol fixation technique incorporated a 15% w/v final concentration of PVA in the incubation solution in order to enhance cellular preservation and to prevent loss by diffusion of soluble material from smeared cells. At this, relatively low, concentration of PVA it was also necessary to fix the cells to retain them on the slide and subsequent studies were therefore made using a PVA concentration/

concentration of 20% w/v, which is close to the maximum solubility which can be achieved at room temperature. Cellular preservation in unfixed films was still unsatisfactory however.

In order to avoid the disruptive effect of the shearing force required to spread a marrow film, a supravital technique, in which a drop of EDTA-anticoagulated marrow was added to the incubation solution containing 20% w/v PVA, was developed. At the completion of the reaction, the incubation solution was centrifuged and a film made from the cell button. This resulted in a considerable increase in the amount of formazan per cell, compared with fixed films, and this was shown to be unrelated to the cell count in the incubation solution over the range 360-3,600 nucleated marrow cells per cu mm.

Cell preservation was poor, however, and higher concentrations of various polyvinyl alcohols of differing molecular weight and acetyl group content (see Table XII) resulted in viscous incubation solutions which were difficult to handle at room temperature and which tended to gel. A collagen polypeptide preparation (derived from collagen by enzymatic degradation) was used as an alternative since concentrations up to 40% w/v could be achieved. This preparation gave slightly better cellular preservation/

TABLE XIIAdditives which Enhance the Preservation of Unfixed Leucocytes

CHEMICAL		MOLECULAR WEIGHT (approx.)
Polyvinyl alcohol (Polyviol)	VO3/140	15,000
	W25/150	80,000
	W40/140	100,000
Collagen polypeptide	5176	15,000
	5177	18,000
	5178	21,000
Ficoll		400,000

preservation than PVA at equivalent concentrations of 5%, 10% and 20%, for incubation periods of 60 min. For incubation periods of 90-120 min a 40% w/v polypeptide concentration gave significantly better preservation, with increased formazan production, than a 20% w/v concentration.

At these relatively high concentrations a difficulty arose in separating the cells from the fairly viscous working solution at the completion of incubation. A number of detergents (Triton X100; 7X; and Tween 40, 60 and 80) at concentrations of 0.1-10% in distilled water, saline and 5% glucose, were used to wash the leucocytes free from incubation solution. A single wash in 2% Tween 80 in 5% glucose gave best results when a 40% concentration of polypeptide was used; a 0.5% concentration of Tween 80 was satisfactory when a lower (20% w/v) concentration of polypeptide was used for 60 min incubation periods. After washing it was possible to achieve better cell preservation by centrifuging the cells directly on to glass slides in a Shandon cyto-centrifuge (600-1,000 rpm for 5 min) as an alternative to smearing the spun deposit.

5.3 Disadvantages of supravital technique using Collagen Polypeptide

(a) Retention of endogenous substrate - supravital techniques/

techniques for dehydrogenase cytochemistry were originally used by de Souza and Kothare (1959); Ackerman (1960); Marcuse and Cochran (1961); and Rozenszajn and Shoham (1967). All of these workers found 'non-specific' endogenous enzyme activity in control preparations lacking substrate (see I.4).

This was also found to be a disadvantage with the cell suspension-cytocentrifuge technique. Moreover, the NBT formazan deposit with this method tends to include a diffuse component, in contrast to the cold ethanol fixation method using MTT as tetrazole, and the combination of significant diffusion together with positive endogenous 'control' preparations invalidates the use of a simple semi-quantitative visual scoring technique based on granule size and number. A scanning and integrating microdensitometer (see IV.7) was therefore used in subsequent experiments to assess formazan production. This confirmed that demonstrable net enzyme activity (total minus endogenous) for G6PD was greater using the cell suspension-cyto-centrifuge technique compared with cold-ethanol fixed films (see Fig. 6).

It is not clear why the MTT-cold ethanol fixation method resulted in an absence of diffuse formazan in the cell cytoplasm compared with subsequent NBT-'no-fix' methods. This/

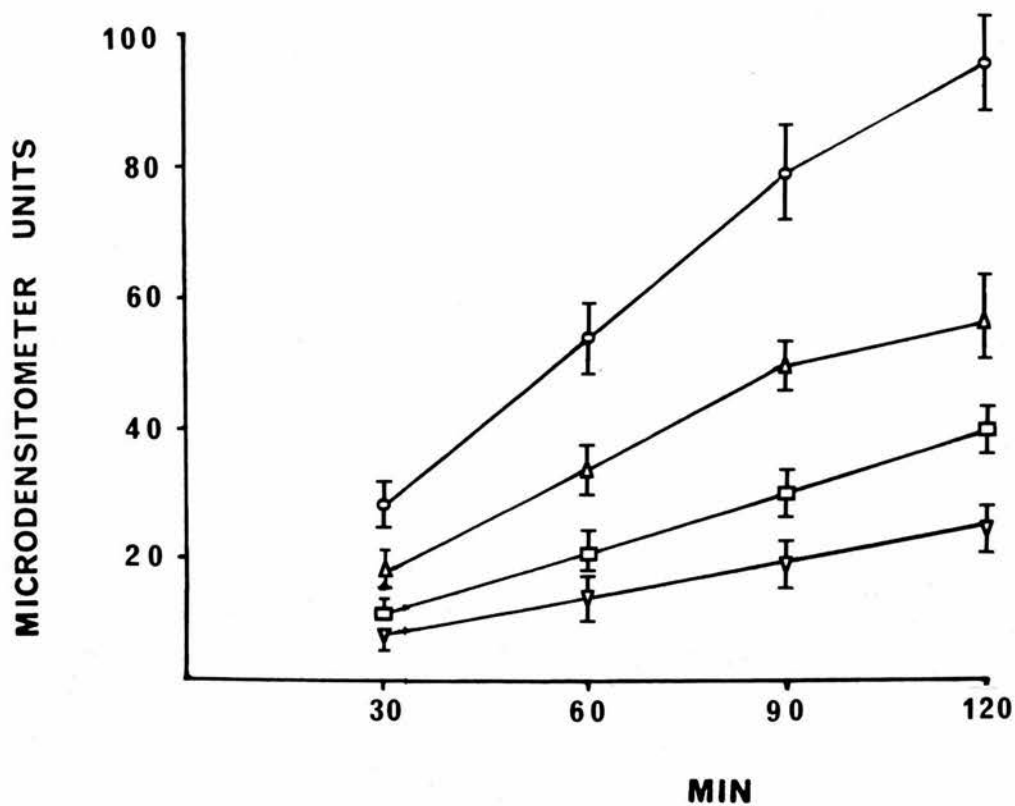


Fig. 6. Activity-time curves showing activity (mean of 50 cells \pm 1 S.D.) of G6PD gross (O), c-NADP (Δ) and G6PD net (\square) in lymphoblasts incubated in suspension, compared with G6PD activity net in cold-ethanol fixed cells (V).

This may have resulted from the action of the fixative rendering the cytoplasmic membrane permeable in respect of the diffuse component, the weaker affinity of MTT for hydrogen and the effect of CoCl_2 on the capture reaction.

(b) Identification of cells - the combination of the cell protective action of the collagen polypeptide, together with cyto-centrifugation, resulted in cells which were slightly smaller, more rounded and less easy to identify compared with conventional smeared films. There was also more variation in the amount of formazan deposit from cell to cell compared with smeared films and this may have arisen due to variations in membrane permeability accentuated by a coating of polypeptide.

(c) Batch variation of polypeptide - subsequent batches of polypeptide showed manufacturing variations resulting in crystalline deposits on the slide overlying the leucocytes and an abnormally acid incubation medium (pH 6.4) but the reason for this remained unknown.

5.4 Supravital technique using Ficoll A significant improvement in the technique was achieved by replacing the collagen polypeptide with an equivalent concentration of Ficoll, which is a synthetic high polymer formed by the copolymerization of sucrose and epichlorhydrin. The hydroxyl/

hydroxyl groups in the molecule ensure very good solubility in aqueous media and thus, unlike sucrose, high density solutions of low viscosity and osmotic pressure can be obtained up to concentrations of 50% w/v.

Sucrose solutions were originally used for cell separation procedures to allow the isolation of nuclei and mitochondria while preserving normal morphology and high enzyme activity (Dixon and Webb, 1964). The protective action of sucrose, and its polymers, is not fully understood although Lehninger (1965) suggested that they 'freeze' mitochondrial structure by inhibiting the swelling and contraction mechanisms which operate in response to variations in intracellular ATP concentration.

Ficoll has been used mainly for the separation and isolation of cells and subcellular particles during centrifugation - for example, in the isolation of mitochondria from hepatic tissues (Hess, Stäubli and Riess, 1965) and the separation of leucocytes from blood (Noble, Cutts and Carroll, 1968; Harris and Ukaejiofo, 1970). Its advantages as a cell preservative during incubation for dehydrogenase cytochemical reactions were found to be several:

(a) It is chemically inert and was stable at room temperature.

(b) /

(b) The high stability allowed high density solutions of low viscosity to be prepared which could easily be pipetted at room temperature.

(c) No batch variations were detected and there was no substantial pH alteration when added to the incubation solutions.

(d) It was readily washed off cells following incubation, thus allowing the leucocytes to remain discrete with a clear background between cells. This aided scoring when using the microdensitometer.

A 40% w/v concentration of Ficoll was found to give optimal cellular preservation for incubation periods of 120 min, and the preservation achieved was slightly better than for the collagen polypeptide at equivalent concentration. The amount of formazan produced was approximately the same for the two preparations. Since several collagen polypeptides, some forms of PVA, and also Ficoll have a cell protective effect during incubation this should be regarded as a non-specific effect shared by several polymers (see Table XII). The secondary benefits of Ficoll, listed above, were the main reasons for the choice of this additive.

5.5 Unfixed film technique using Ficoll The cell protective effect and ease of handling of solutions containing a high concentration of Ficoll also made it possible to study unfixed marrow films. When conventional, unfixed, air-dried/

air-dried films were incubated in working solutions containing a 40% w/v concentration of Ficoll good leucocyte preservation was achieved and, at the completion of incubation, the Ficoll solution could readily be drained from the slides. An almost invariable loss of erythrocytes occurred (see Fig. 7) but this facilitated subsequent scoring by microdensitometry by providing a clear background between cells.

It was advantageous to wash the remaining Ficoll from the slide, and also accurately terminate the enzyme reaction, by flooding the slide with a jet of 40% formalin from a wash bottle.

The unfixed marrow film technique was found to have several advantages over the cell suspension-cyto-centrifuge method:

(a) Individual cells appeared larger and more flattened; this facilitated identification.

(b) The endogenous enzyme activity was considerably less. This represents non-specific enzyme activity due to the retention of endogenous substrate and requires to be subtracted from the test activity, in the presence of specific substrate, in order to obtain net enzyme scores. It is therefore preferable to obtain low endogenous values in/

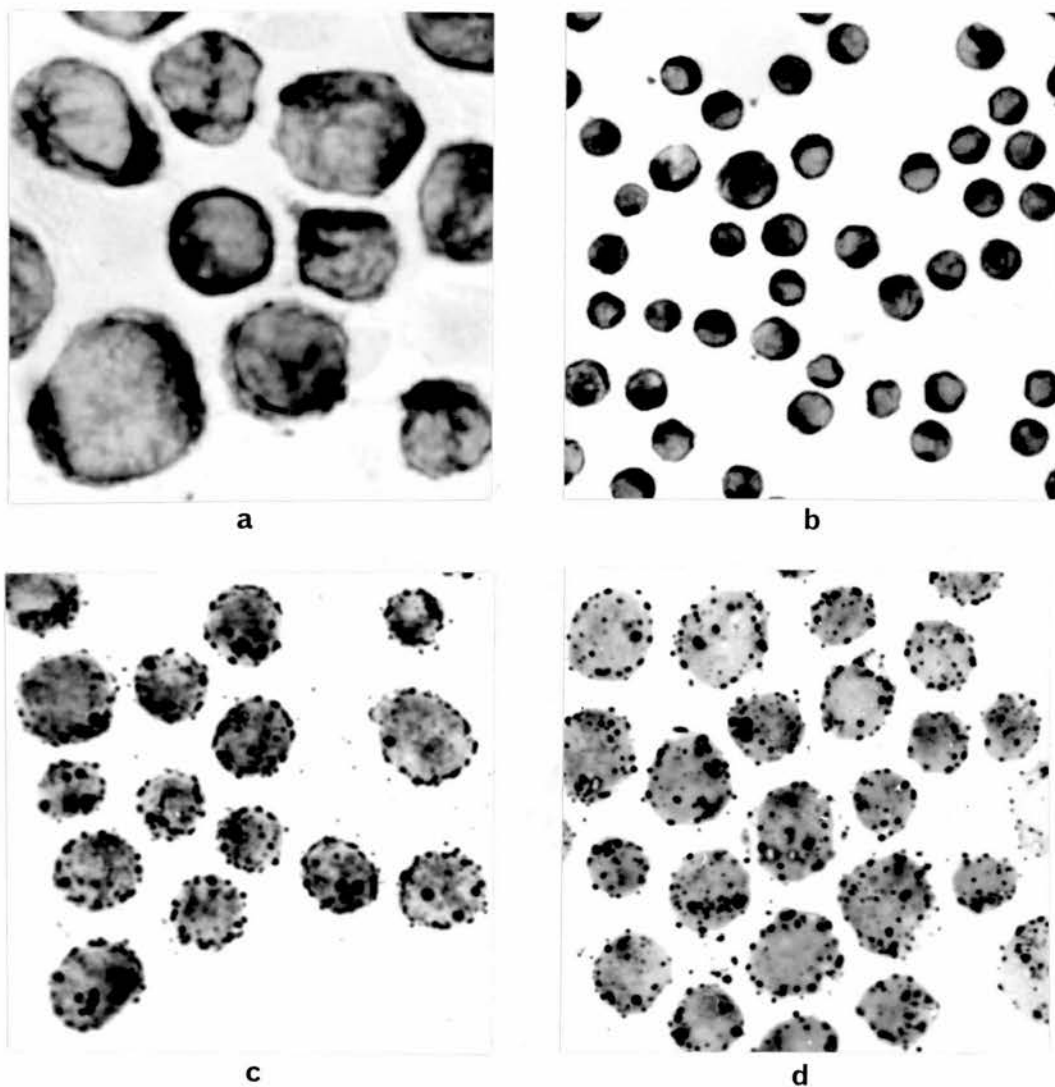


Fig. 7. Dehydrogenase enzyme activity in leukaemic lymphoid cells counterstained with methyl green.

- a. LD - acute leukaemia lymphoblasts. x 1600.
- b. LD - chronic leukaemia lymphocytes. x 864.
- c. MD - acute leukaemia lymphoblasts. x 864.
- d. β -HBD - acute leukaemia lymphoblasts. x 864.

in order to avoid the possibility of false low net enzyme scores.

(c) The formazan deposit was found to be more uniform in amount from cell to cell (see Fig. 7).

These advantages are probably derived from the lateral shearing force required to spread marrow and blood films. This shear action converts the normally globular-shaped leucocyte into a flattened form stretched out on a glass slide, and this must have a disruptive effect on the cytoplasmic membrane. This may be sufficient to allow diffusion from the cell of some endogenous substrate; at the same time it may allow easier access of extrinsic substrate and co-enzyme to the cell's enzyme systems.

In order to demonstrate that there was no simultaneous loss of enzyme by the same route, the unfixed film technique was carried out simultaneously with the cell suspension-cyto-centrifuge method using the same incubation solutions incorporating a 40% w/v concentration of Ficoll. The soluble, cytoplasmic enzyme G6PD and its equivalent control (c-NADP) were studied in blast cells from eight acute leukaemia marrows for an incubation period of 90 min (see Table XIII). The mean figure for net enzyme activity in/

in the unfixed film preparations was 39.9 ± 14.6 compared with 30.3 ± 12.5 in cells incubated in suspension. The higher net value in the smeared cells is presumably due to the lower endogenous, control activity, but this increase was not significant ($P > 0.5$). Thus it would appear there was no significant loss of this soluble enzyme from the smeared cells.

5.6 Dialysis of endogenous substrate An attempt was made to dialyse out endogenous substrate in order to achieve near negative control slides. Enzymes are non-dialysable because of their colloidal nature (Staunton West et al., 1966). Prior to incubation using the cell suspension-cyto-centrifuge technique, 2 drops of anti-coagulated marrow were added to each of 1 ml of 0.005M and 1 ml of 0.015M pyrophosphate buffer (Dhungat and Sreenivasan, 1954) for periods of 1 hour and 2 hours at 20°C , and for 24 hours at $0-4^{\circ}\text{C}$. The remaining marrow cells were stored at $0-4^{\circ}\text{C}$ in the original EDTA tube, then processed with the dialysed cells for LD and c-NAD. There was no detectable difference in formazan formation for either the test or control preparations. Wagner et al. (1956) also failed to dialyse out the substrates responsible for endogenous oxygen consumption using this technique and required to dialyse against distilled water for/

TABLE XIII

Comparison of Net Enzyme Activity (Gross Less Control)
for G6PD in Unfixed Smearred Cells Compared with Cells
Incubated in Suspension in Eight Acute Leukaemia Marrows.
Values Expressed in Arbitrary Microdensitometer Units

SMEARED CELLS			CELLS IN SUSPENSION		
G6PD (GROSS)	c-NADP	G6PD (NET)	G6PD (GROSS)	c-NADP	G6PD (NET)
55.7	8.8	46.9	65.5	39.6	25.9
76.9	23.3	53.7	97.4	48.8	48.6
67.2	14.9	52.3	78.3	34.1	44.2
38.0	12.4	25.6	47.7	36.3	11.4
65.9	12.3	53.6	115.2	88.8	26.4
59.6	21.1	38.5	122.4	93.1	29.3
26.5	13.0	13.5	40.5	21.0	19.5
56.3	20.7	35.6	77.5	40.0	37.5
Mean (\pm SD)		39.9 \pm 14.6	Mean (\pm SD)		30.3 \pm 12.5

for 18 hours in order to achieve this. In the experiments described above this resulted in cell disruption.

A similar study was carried out for marrow films. Unfixed films were immersed for periods of 1, 5 and 10 min in normal saline (Gough and Elves, 1967); 0.05M glycylglycine buffer; and 0.005M and 0.015M pyrophosphate buffer (Dhungat and Sreenivasan, 1954). There was considerable loss of cells from the films unless a 40% w/v concentration of Ficoll was incorporated in the dialysing solution. In the presence of added Ficoll cell loss was much less, but still substantial, and although there was slight reduction in formazan production this occurred equally in the test and control preparations. In view of the associated deterioration in cell preservation this refinement was not considered to be of significant value.

5.7 Unfixed v. acetone-fixed films When acute leukaemia marrow films were fixed for 30 sec in 60% acetone at room temperature prior to incubation in the presence of Ficoll, there was a significant improvement in the preservation of erythrocytes compared with unfixed films, but there was a constant reduction (approximately two-fold) in demonstrable enzyme activity for both intra- and extra-mitochondrial enzymes (see Figs. 8 and 9). Therefore any theoretical/

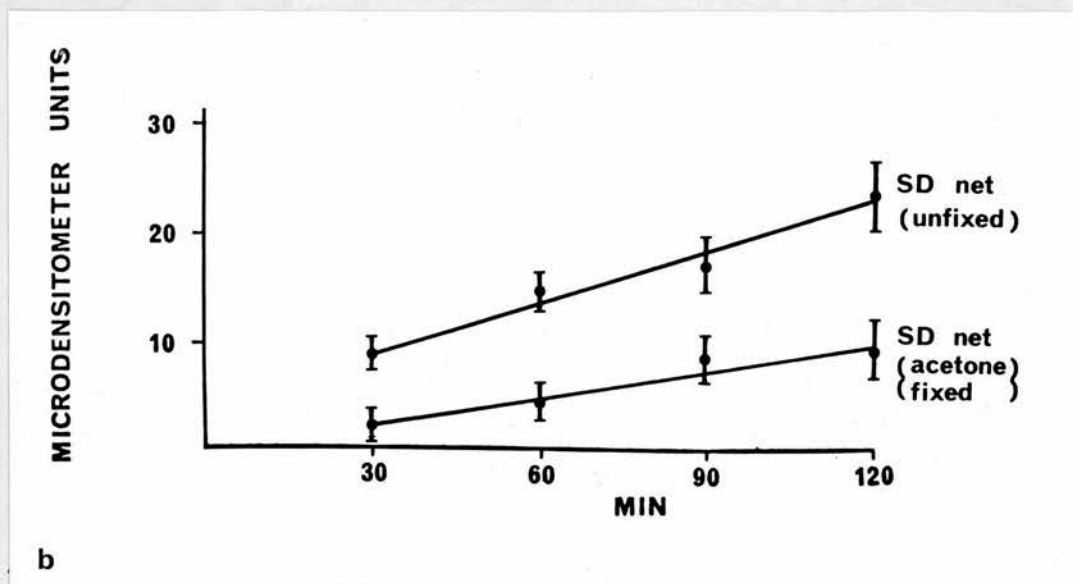
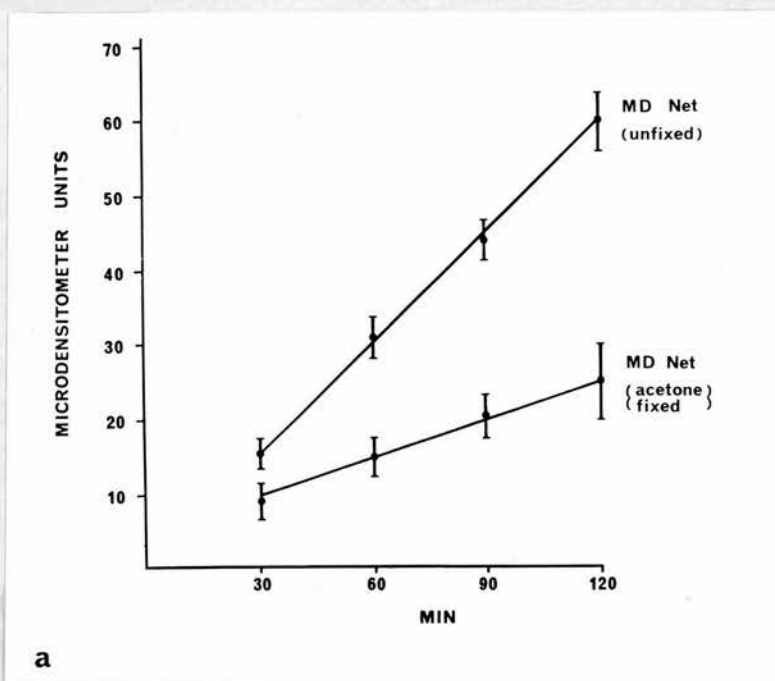


Fig. 8. Comparison of net enzyme activity (mean of 50 cells \pm 1 S.D.) in lymphoblasts when unfixed, and when fixed with 60% acetone for 30 sec at room temperature, prior to incubation in the appropriate reaction medium containing a 40% w/v concentration of Ficoll. a. MD; b. SD

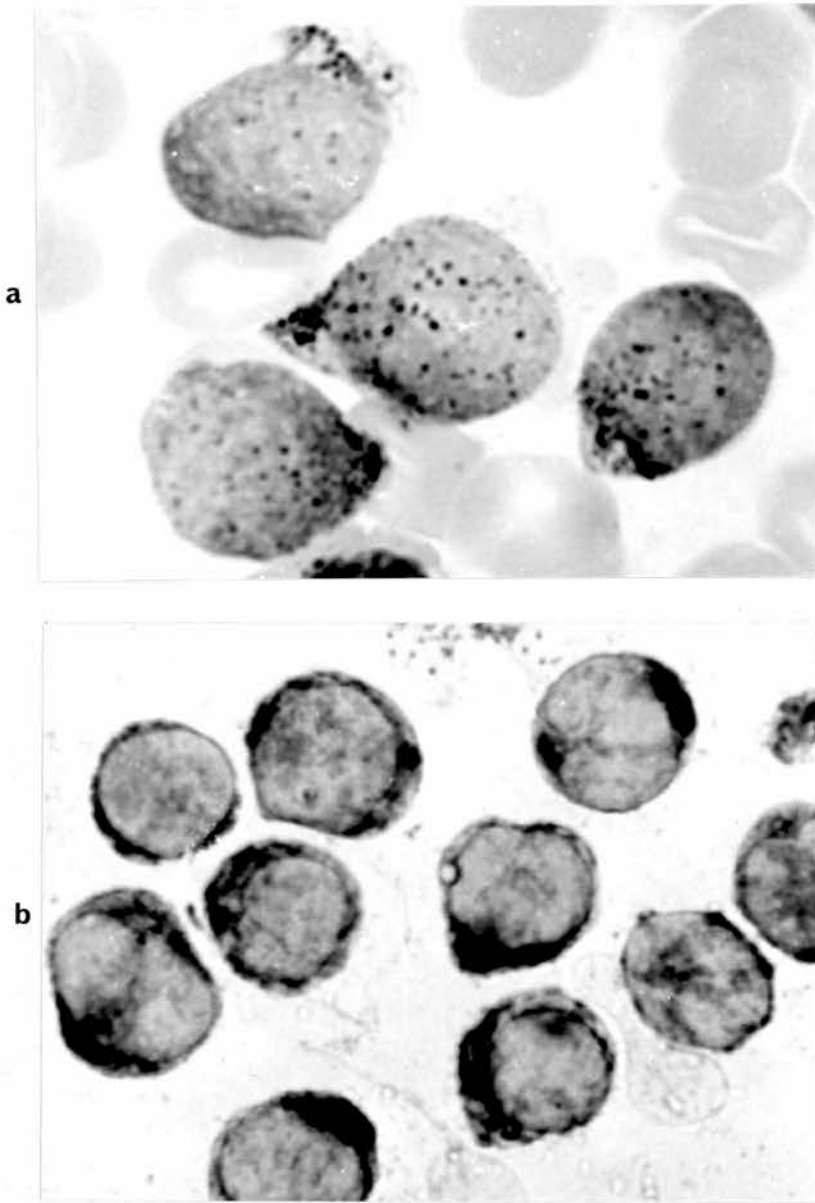


Fig. 9. G6PD activity in lymphoblasts following incubation for 90 min in the appropriate reaction medium containing a 40% w/v concentration of Ficoll. Counterstained with methyl green. x 1600.
a. acetone fixed; b. unfixed

theoretical beneficial effect of acetone fixation, by virtue of a lipid-clearing effect which might increase membrane permeability and also prevent false localisation of NBT-formazan (Quaglino and Hayhoe, 1960), was more than offset by its inhibitory effect on enzyme activity. Baillie, Ferguson and Hart (1966) obtained a similar result from a study of hydroxysteroid dehydrogenase activity.

5.8 Effect of refrigeration on films Refrigeration at -20°C has been shown to increase membrane permeability and penetration of substrate, and thus enhance the demonstrable activity of lysosomal enzymes (Lorbacher, Yam and Mitus, 1967). Unfixed marrow films were therefore refrigerated at -20°C for 60 min, prior to incubation in the presence of added Ficoll, and compared with control films maintained at room temperature during the 60 min period. Refrigeration resulted in a deterioration in cell preservation with a slight decrease in formazan production, probably as a result of this. It was not possible to enhance the activity of either extra- or intra-mitochondrial enzyme activity by this procedure and it was presumed that the shearing force required to spread the cells resulted in sufficient membrane damage to permit adequate penetration of substrates of low/

low molecular size.

6. Incubation Technique and Composition of Working Solutions

6.1 Incubation technique A thermostatically controlled incubator lined with polystyrene and with a sliding perspex door for ease of access was designed to provide accurate temperature control ($37 \pm 1^{\circ}\text{C}$) during the processing of large numbers of marrow films (see Fig. 10). Two perspex incubation-humidity boxes were also designed to fit within the incubator in order to provide additional insulation against temperature fluctuation, to maintain high humidity and thus prevent evaporation, and to allow individual adjustments of the gaseous atmosphere. Humidity was maintained by placing a small beaker of distilled water in each compartment.

An open-ring technique (Chayen et al., 1969) was used to provide individual incubation solutions for each slide and to allow the simultaneous processing of a large number of slides. Individual perspex rings (1.5 x 0.5 cm) were fixed by a thin smear of paraffin wax just proximal to the tail of air-dried films and filled with incubation solution. It was not found necessary to seal the ring with a coverslip since no significant evaporation occurred during incubation. Trays of films were then inserted in the pre-warmed incubation-humidity boxes and incubation carried out/



Fig. 10. Incubation-humidity box within polystyrene-lined incubator showing shelves designed to hold trays of slides and attachments to allow incubation in an atmosphere of nitrogen. The heating element is seen to the rear of the incubator.

out in an atmosphere of nitrogen. The nitrogen was bubbled through a Wolff bottle containing distilled water at 37°C in order to avoid lowering of the incubation temperature.

At the completion of incubation the perspex rings were pulled off, the remaining Ficoll washed off and the reaction terminated in a 40% solution of formalin, and the slides counterstained for 10 min using 2% aqueous methyl green. The slides were then allowed to air-dry before storing in light-tight boxes prior to scoring.

6.2 Duration of incubation A standardised incubation time of 90 min was chosen for all the enzyme reactions to simplify processing. This period was chosen since longer incubation times resulted in too dark a formazan deposit with the stronger reacting enzymes (LD and MD) for the microdensitometer to record. It was sufficiently long for the weaker reacting enzymes (α -GPD, SD and GD) to produce sufficient formazan for evaluation. Incubation for this duration also ensures linear oxidation of substrate (Dhungat and Sreenivasan, 1954; and see Figs. 11, 12 and 13) and provides a significant differential between exogenous and endogenous enzyme activity (see Figs. 11, 12 and 13).

6.3 Choice of buffer and incubation pH Phosphate and Tris/

Tris buffers have been widely used for dehydrogenase cytochemistry although the choice does not appear to be critical provided that the pH is maintained within the range 7.0-7.4 throughout the period of incubation. There is some evidence, from histochemical studies, that the considerable loss (70%) of nitrogenous material from tissue sections immersed in phosphate buffer (Jones et al., 1963) is reduced (to 50-60%) when glycylglycine is used as the buffer (Altmann and Chayen, 1965). Although this confers only a marginal benefit, the latter buffer has now been used extensively in dehydrogenase histochemical reactions (Chayen et al., 1969).

The use of 0.05M glycylglycine buffer pH 7.6 was found to provide sufficient buffering activity to maintain a final pH of 7.3-7.4 throughout the 90 min incubation period in this study.

6.4 Choice of tetrazolium salt NBT was selected in preference to MTT in this section since it has the advantages of high redox potential and substantivity for protein and produces a stable formazan of dark colour which has little aqueous solubility. It also produces better localization than the method of Pearse (1957) using MTT and cobalt (Novikoff, Shin and Drucker, 1961), although/

although this was not an important requirement for this study. The dark-coloured formazan deposit contained both diffuse and particulate components and was suitable for quantitation by microdensitometry. A final concentration of NBT of 1 mg/ml was chosen - no increase in formazan production was obtained for any of the enzymes at a concentration of 5 mg/ml.

6.5 Omission of metallic ion activators These are not required to prevent formazan crystallisation when NBT is used as the tetrazole and, since it was also found unnecessary to use them as activators of the weakly reacting dehydrogenases in the no-fix technique, they were not added to the incubation solutions. Rozenszajn and Shoham (1967) also found no increase in NBT-formazan production in the presence of Al^{3+} and Co^{2+} and Baillie et al. (1966) found no increase in the presence of Mg^{2+} .

6.6 Addition of calcium chloride Ca^{2+} are reported to have a favourable effect during nuclear isolation procedures (Schneider and Petermann, 1950) and also stabilize nucleoli (Georgiev, 1967) and cause protoplasmic gelling (Heilbrunn, 1943). Ca^{2+} are also taken up by mitochondria during respiration (Vasington and Murphy, 1962; Lehninger, Rossi and Greenawalt, 1963) and can result/

result in stimulation of mitochondrial respiration (Rossi and Lehninger, 1964). For these reasons, calcium chloride ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) was incorporated in all reaction media to protect unfixed cells, although no critical evaluation of its usefulness in stabilising cell structure was made.

6.7 Co-enzyme concentrations A final concentration of 1 mg/ml for both NAD and NADP was used, the concentration for NADP being taken from Chayen et al. (1969). Erhart, Hormann and Armbruster (1969) have recently described increased levels of NAD in the chronic lymphocytic leukaemia leucocyte, with normal levels in the lymphoblast, and Beck and Valentine (1952) had previously reported a higher than normal NAD requirement for maximum glycolysis in chronic lymphatic leukaemia. There was, however, no detectable increase in formazan production (for a 90 min incubation period) for LD, α -GPD-NAD, β -HBD and MD in lymphoblasts in the presence of a five-fold increase in concentration of NAD. A similar result was obtained for G6PD in the presence of NADP, and a final concentration of 1 mg/ml was therefore adopted for both co-enzymes.

6.8 Substrate concentrations A substrate concentration of/

of 0.05M was used initially for all dehydrogenases, with the exception of G6PD for which a concentration of 0.005M was used as recommended by Chayen et al. (1969). These concentrations were satisfactory apart from the reactions for α -GPD and α -GPD-NAD which showed stronger activity when a final substrate concentration of 0.1M was used. Linear activity-time curves were obtained for net activity of each of the enzymes using these concentrations (see Figs. 11, 12 and 13) and no further increase in formazan production was obtained with a five-fold increase in substrate concentration.

6.9 Preparation of reaction solutions The final composition of the individual reaction solutions is given in Table XIV. Fresh reagents were prepared for each experiment. The required total amount of glycylglycine buffer, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and NBT were first mixed and Ficoll then added; dissolution of the latter was aided by a magnetic stirrer. The individual substrate and co-enzyme concentrations were weighed out into separate glass beakers and the buffer- $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ -NBT-Ficoll mixture added immediately prior to incubation. Care was taken to ensure adequate mixing of the final working solutions without carry-over.

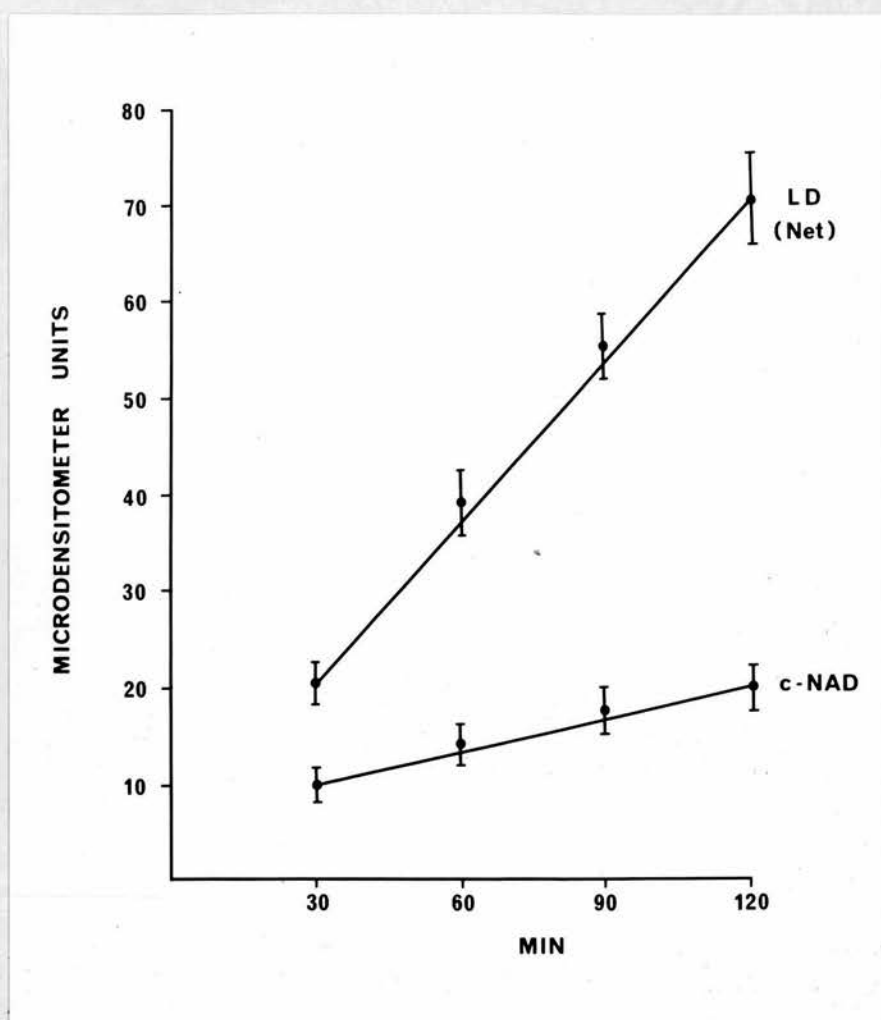


Fig. 11a. Activity-time curves for net enzyme activity (mean of 50 cells \pm 1 S.D.) of the extramitochondrial dehydrogenase LD in lymphoblasts, together with the appropriate control activity (c-NAD) obtained in the absence of added exogenous substrate.

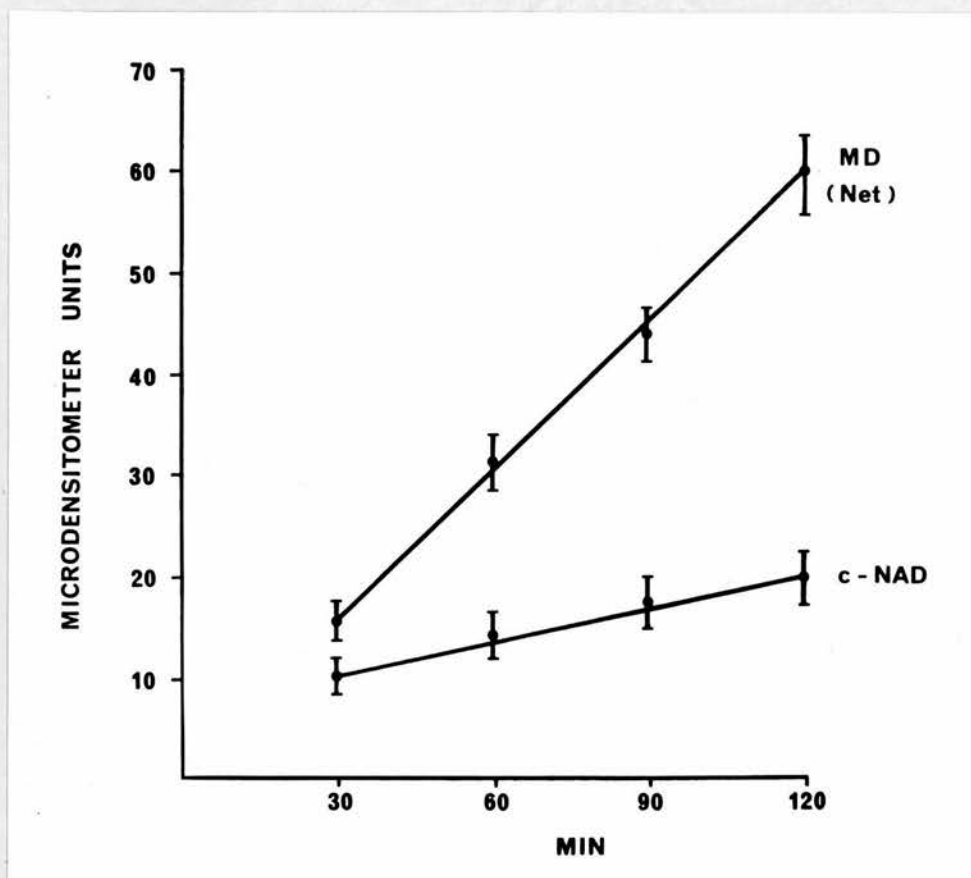


Fig. 11b. Activity-time curves for net enzyme activity (mean of 50 cells \pm 1 S.D.) of the extramitochondrial dehydrogenase MD in lymphoblasts, together with the appropriate control activity (c-NAD) obtained in the absence of added exogenous substrate.

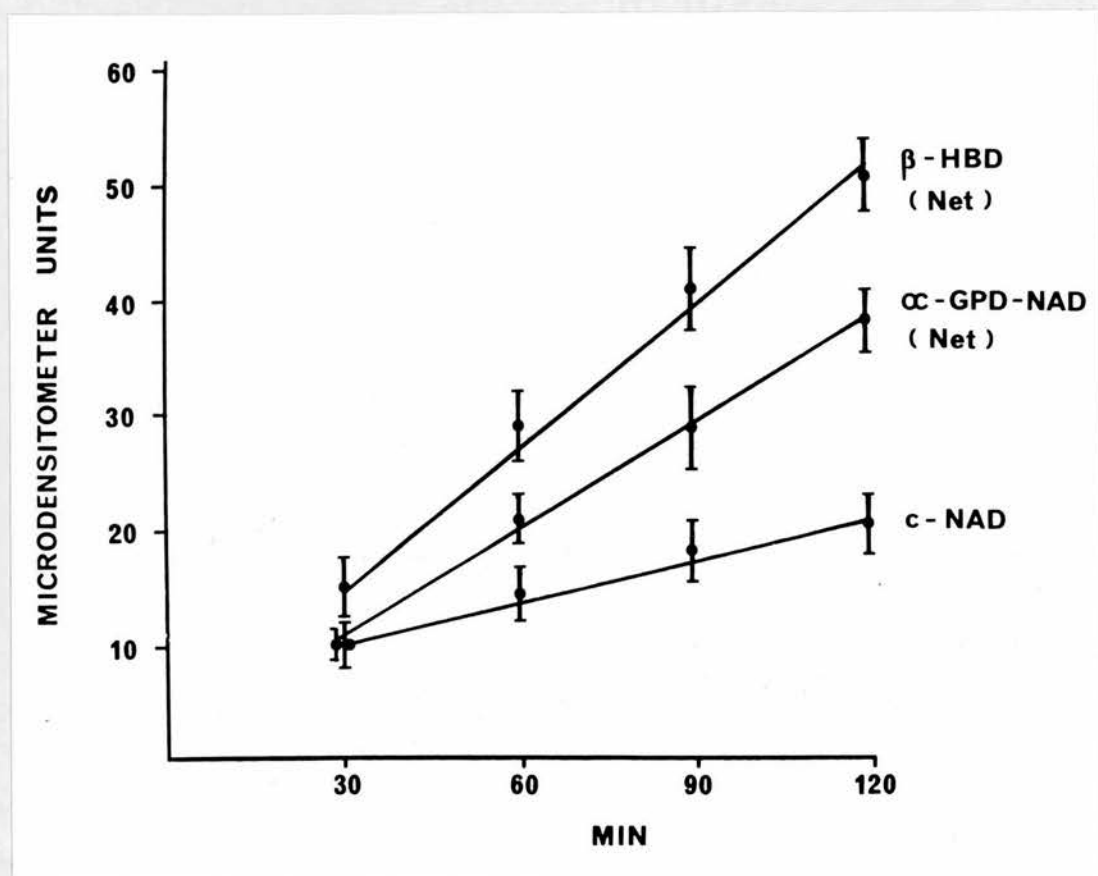


Fig. 11c. Activity-time curves for net enzyme activity (mean of 50 cells \pm 1 S.D.) of the extramitochondrial dehydrogenases β -HBD and α -GPD-NAD in lymphoblasts, together with the appropriate control activity (c-NAD) obtained in the absence of added exogenous substrate.

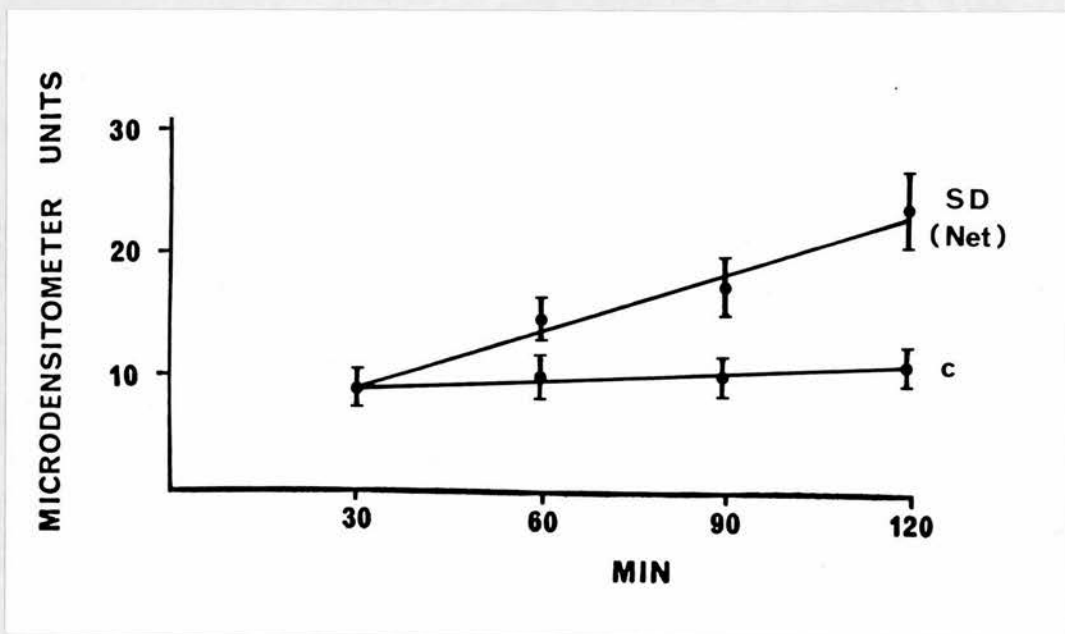


Fig. 12. Activity-time curves for net enzyme activity (mean of 50 cells \pm 1 S.D.) of the intramitochondrial dehydrogenase SD in lymphoblasts, together with the appropriate control activity (c) obtained in the absence of exogenous substrate.

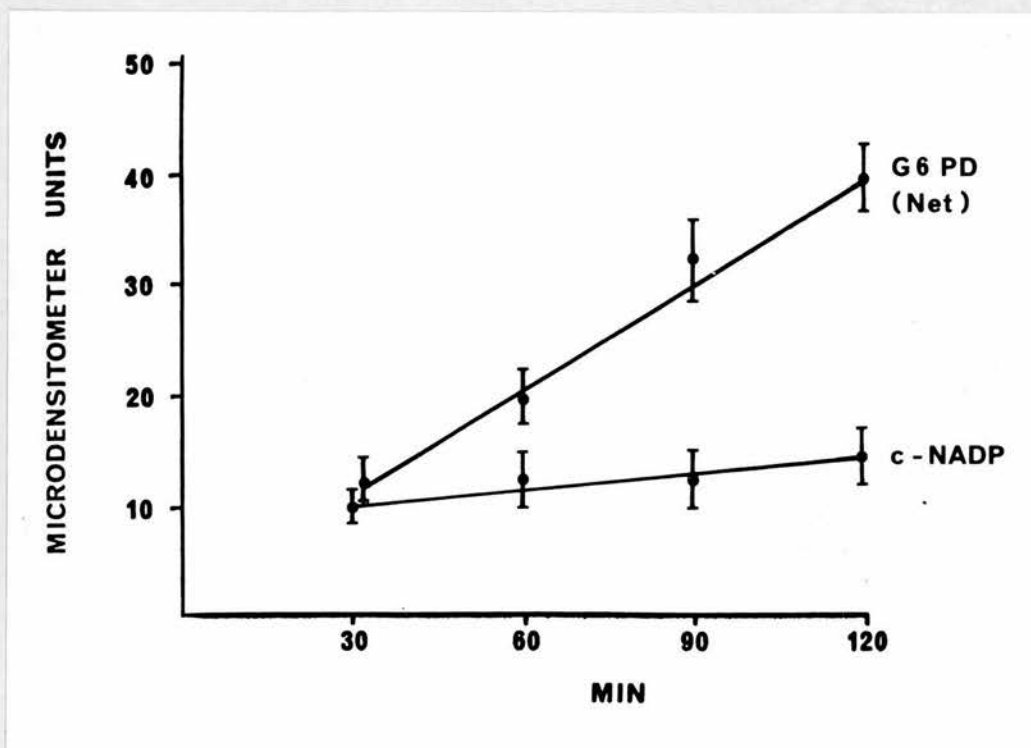


Fig. 13. Activity-time curves for net G6PD activity (mean of 50 cells \pm 1 S.D.) in lymphoblasts, together with the appropriate control activity (c-NADP) obtained in the absence of added exogenous substrate.

TABLE XIV

Composition of incubation solutions for 'no-fix' technique

	IAD	α -GPD-NAD	β -HBD	MD	GPD	α -GPD	SD	G6PD	C-NAD	C-NADP	C
Substrate*	0.02 ml	108 mg	63 mg	39 mg	43 mg	108 mg	68 mg	6 mg	-	-	-
Buffer (ml)	5	5	5	5	5	5	5	5	5	5	5
NBT (mg)	5	5	5	5	5	5	5	5	5	5	5
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (mg)	65	65	65	65	65	65	65	65	65	65	65
Ficoll (g)	2	2	2	2	2	2	2	2	2	2	2
NAD (mg)	5	5	5	5	5	-	-	-	5	-	-
NADP (mg)	-	-	-	-	-	-	-	5	-	5	-

* See 'Source List of Chemicals and Equipment'

7. Application of Scanning and Integrating Microdensitometry

Visual, semi-quantitative scoring techniques have been widely used to assess the amount of reaction end-product in cytochemical reactions. When the reaction end-product consists of a mixture of granules and diffuse positivity, however, the eye tends to be attracted to selected areas of strong positivity and it becomes difficult to integrate the total amount of reaction product in the cell. Also, it becomes difficult to maintain an objective standard scoring technique over a period of months, and accuracy is further impaired when it becomes necessary to score control reactions and subtract these values to achieve net enzyme scores.

A scanning and integrating microdensitometer (Barr and Stroud, type GN2) was therefore used to obtain a more objective and accurate assessment of the reaction end-product. The basic principle of microdensitometry is similar to that of spectrophotometry except that the quartz cell of the spectrophotometer is replaced, in this case, by a biological cell smeared on to a glass slide. A conventional microscope (see Fig. 14) is used to compensate for the reduction in size and allows an enlarged version of the cell to be projected on to a photo-cell. Since the formazan in these reactions is distributed/



Fig. 14. Barr and Stroud (type GN2)
scanning and integrating microdensitometer.

distributed irregularly throughout the cell, in contrast to the chromophore in spectrophotometry which is present in homogeneous solution, a scanning grid system is used to ensure that a small, relatively homogeneous part of the cell is being measured at any given time. The individual grid readings are then computed and integrated electronically to give a visual numerical display of the final value.

A green nuclear counterstain was chosen to provide a contrast with the dark-coloured formazan, and micro-densitometer readings were taken in green light (550 nm) which was absorbed almost maximally by the formazan but not appreciably by the methyl green. This ensured that variations in nuclear size and mass had a minimal effect, compared with the formazan deposit, on the final reading for individual cells.

A standardised light intensity was achieved by taking a blank reading in the clear area adjacent to each cell and then adjusting the light intensity to give a value between 5-15 divisions on the relative absorption scale; the value for this was then subtracted from that obtained for the cell. This compensated for any variations in intensity of the methyl green counterstain. The cell was then visualised using a X100 oil immersion objective/

objective and scored using a constant size of field stop suitable for both blast cells and small lymphocytes. It was not found practical to adjust microdensitometer readings in relation to cell area since not only did the individual cell size vary but also the relative proportion of the cell area taken up by the nucleus varied in different cell types. The use of green light together with a green counterstain minimised these variations and the subtraction of a mean control value for cells of the same size provided a further safeguard.

The following microdensitometer settings were also standardised:

extinction	- 1.0
absorption range	- X20-100
photomultiplier setting	- 1
projection lens setting	- high
collector lens setting	- X100

8. Calculation of Net Enzyme Activity

A mean figure for 20-50 cells was obtained for each test film incubated in the presence of added substrate and was recorded as gross enzyme activity. Control films, incubated in the absence of substrate and co-enzyme (c), or in the absence of substrate but in the presence of appropriate/

appropriate co-enzyme (c-NAD; c-NADP), were included with each marrow studied. The films for c and c-NADP invariably showed little or no formazan production (see Figs. 12 and 13) and the microdensitometer readings obtained (usually 5-15 units) largely represented the effects of the counterstained nuclear mass. The c-NAD preparations (see Fig. 11) consistently showed more formazan production (usually 10-20 units) and this probably represented the action of the NAD-dependent dehydrogenases on the small amount of endogenous substrate retained within the cell. The mean values for 20-50 cells were determined for each of the control preparations and subtracted from the gross scores for the appropriate enzymes to give net values. All values obtained were in arbitrary microdensitometer units.

9. Reproducibility of the Technique

One acute leukaemia relapse marrow specimen was processed 10 times on the same day, using separate incubation solutions, for the enzyme LD and a control preparation lacking substrate (c-NAD). Fifty blast cells were scored in each of the 10 slides for LD and c-NAD and the mean scores (\pm standard deviation) for net enzyme activity determined (see Table XV). This gave a co-efficient/

TABLE XV

Enzyme Scores (mean \pm SD) for 10 Separate Estimations on a Single Leukaemic Marrow Specimen

	LD GROSS	C-NAD	LD NET
Mean score (10)	110.1	13.1	97.0
\pm SD	± 4.8	± 2.1	± 3.9
Co-efficient of Variation	-	-	4.1%

co-efficient of variation of 4.1% for LD net.

10. Patients Studied

Bone marrow specimens were obtained from both children and adults with acute leukaemia and from adults with chronic leukaemia. In the majority of cases the specimen was the initial diagnostic marrow but in the remaining cases marrow was obtained at a time of haematological relapse when resistance had developed to maintenance chemotherapy; specimens from patients who were potentially responsive to a recent change in chemotherapy were not included. A blood, rather than a marrow, specimen was studied in a few patients with a high leucocyte count - in two of these patients, with acute lymphoblastic leukaemia, there was no significant difference between the enzyme activity of marrow and blood blast cells.

Cytochemical confirmation of the cell type in patients with acute leukaemia was obtained by means of the periodic acid-Schiff and Sudan Black reactions (Hayhoe et al., 1964) and the β -glucuronidase reaction (Mann, Simpson, Munkley and Stuart, 1971). The cytoplasmic distribution of the Sudan Black reaction end-product was the main criterion for differentiation of acute myeloblastic/

myeloblastic leukaemia from monocytic and myelomonocytic types.

The normal control marrows represent specimens aspirated from hospital in-patients who were found to have a predominantly normal myelogram, with no abnormality of the lymphoid or myeloid cell series, and who did not subsequently develop leukaemia.

11. Results

11.1 Enzyme activity in lymphoid cells

11.1.1 Marrow lymphocytes The enzyme pattern for marrow lymphocytes from ten normal children (mean age 6.8 years) was compared with that obtained from ten children in the remission state of acute lymphoblastic leukaemia while receiving maintenance chemotherapy (see Table XVI).

There was no significant ($P > 0.05$) difference between the two groups apart from a lower G6PD activity ($P < 0.05$) in the leukaemic patients. This may represent a suppressant effect of antimitotics on nucleoprotein metabolism and therefore pentose phosphate shunt activity. In subsequent comparisons with leukaemic blast cells the mean figures for these two groups were amalgamated, with the exception of G6PD.

11.1.2 Atypical mononuclears In order to determine the effects on enzyme activity of the virus transformation of lymphocytes/

TABLE XVI

Mean Figures (\pm SD) for Small Lymphocytes from 10 Leukaemic Marrows
In Remission and 10 Non-leukaemic Marrows

	LD	α -GPD-NAD	β -HBD	MD	GD	α -GPD	SD	G6PD
Leukaemia in remission	37.8 \pm 4.9	26.9 \pm 7.3	24.9 \pm 6.1	38.8 \pm 6.6	18.3 \pm 4.4	9.3 \pm 3.7	16.1 \pm 4.7	18.7 \pm 6.9
Non-leukaemic patients	34.3 \pm 4.8	21.7 \pm 5.5	20.0 \pm 4.6	35.3 \pm 4.6	14.2 \pm 4.4	9.9 \pm 2.6	13.5 \pm 3.8	26.4 \pm 9.0
Significance of difference of means (P)	>0.1	>0.05	>0.05	>0.1	>0.05	>0.5	>0.1	<0.05

lymphocytes to the atypical mononuclear cells of infectious mononucleosis, leucocyte concentrates were prepared from the blood of eight patients with Paul-Bunnell, or Monospot, positive infectious mononucleosis. The leucocyte concentrates were prepared either from microhaematocrit buffy coat preparations or by leucocyte separation using a mixture of Ficoll and Triosil (Harris and Ukaejiofo, 1970). The mean scores were compared with the scores obtained for ten normal, young, adult volunteers, but no significant ($P > 0.1$) difference was found (see Table XVII).

11.1.3 PHA-transformed lymphocytes Lymphocytes were obtained from five normal adult volunteers, free from recent illness, by dextran sedimentation of heparinized blood. Duplicate suspensions of the leucocyte concentrate were made in 199 culture medium (Burroughs Wellcome) containing 20% human serum, and PHA (Burroughs Wellcome) added to one of the suspensions. The leucocyte suspension without PHA was incubated for 60 min, spun on to glass slides in a cyto-centrifuge, and its enzyme activity determined. The duplicate suspension was treated in the same way after incubation in the presence of PHA for 72 hrs. A significant ($P < 0.05$) increase in activity/

TABLE XVII

Mean Figures (\pm SD) for Enzyme Scores in Blood Lymphocytes in Chronic Lymphocytic Leukaemia and Infectious Mononucleosis Compared With Normal Small Lymphocytes

	LD	α -GPD-NAD	β -HBD	MD	GD	α -GPD	SD	G6PD
1. Normal adult blood lymphocyte (10)	46.7 ± 6.6	22.9 ± 7.6	16.9 ± 5.5	40.5 ± 4.7	15.1 ± 3.9	12.6 ± 2.9	17.5 ± 2.6	14.1 ± 2.3
2. Chronic lymphocytic leukaemia lymphocyte (17)	67.6 ± 16.5	17.1 ± 7.1	18.6 ± 7.7	60.6 ± 16.4	15.0 ± 10.3	13.1 ± 5.4	17.1 ± 4.5	17.7 ± 7.6
3. Atypical mononuclear (8)	48.3 ± 7.4	19.1 ± 3.3	17.1 ± 5.1	40.9 ± 7.7	13.5 ± 6.0	12.2 ± 3.9	16.7 ± 4.7	17.5 ± 9.4
Significance of difference of means (P)	$1 < 0.01$ $2 > 0.5$ $3 > 0.5$	$1 > 0.05$ $2 > 0.1$	$1 > 0.5$ $2 > 0.5$	$1 < 0.01$ $2 > 0.5$	$1 > 0.5$ $2 > 0.5$	$1 > 0.5$ $2 > 0.5$	$1 > 0.5$ $2 > 0.5$	$1 > 0.05$ $2 > 0.5$

activity of cell enzymes was observed in the PHA-transformed cells (see Table XVIII).

11.1.4 Leukaemic lymphoblasts Marrow or blood specimens from 40 children with acute lymphoblastic leukaemia were studied and 50 cells scored for each enzyme in each marrow. When a mixed cell population of lymphoblasts, with respect to cell size, was present (see Fig. 15) then only the larger cells were scored to avoid inclusion of small and large lymphocytes which might not be derived from the leukaemic cell line. It was not possible to study all the enzymes on each occasion due to the limited amount of marrow available and the actual number of patients studied for each enzyme is stated in Table XIX. When the mean scores were compared with the mean scores obtained for marrow lymphocytes in 20 normal children then there was no significant ($P > 0.1$) difference in activity of the wholly intra-mitochondrial enzymes GD, α -GPD and SD. The blast cells, however, showed a significant ($P < 0.05$) increase in activity of those enzymes which are either wholly extra-mitochondrial (LD) or are believed to have an extra-mitochondrial action (MD and β -HBD), with the exception of the α -glycerolphosphate shuttle enzyme α -GPD-NAD and the pentose phosphate shunt enzyme G6PD.

11.1.5/

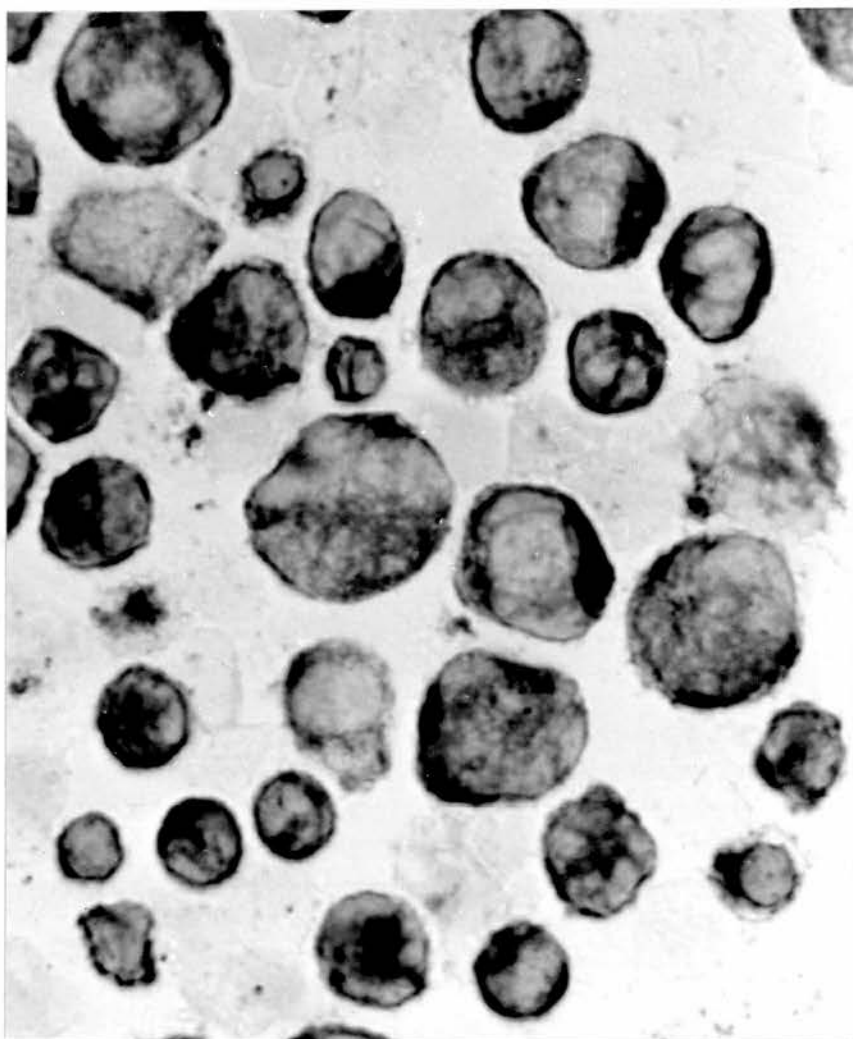


Fig. 15. LD activity in mixed cell population, in respect of cell size, in acute lymphoblastic leukaemia. Methyl green counterstain. x 1600.

TABLE XVIII

Mean Figures (\pm SD) for PHA-transformed lymphocytes from five normal adults; fifty lymphocytes or PHA-cells scored for each enzyme for each adult

	LD	α -GPD-NAD	β -HBD	MD	GD	α -GPD	SD	G6PD
Small lymphocytes (1 hr)	28.7 ± 4.5	15.8 ± 4.2	12.6 ± 7.3	32.3 ± 5.3	12.2 ± 3.6	8.2 ± 1.2	14.1 ± 2.2	17.3 ± 1.4
PHA-transformed lymphocytes (72 hr)	120.5 ± 21.3	43.0 ± 7.9	46.2 ± 10.4	101.7 ± 18.7	41.6 ± 27.1	22.1 ± 5.4	37.7 ± 7.9	53.8 ± 6.6
Significance of difference of means (P)	< 0.01	< 0.01	< 0.01	< 0.05	< 0.05	< 0.01	< 0.01	< 0.01

TABLE XIX

Mean Figures (\pm SD) for Enzyme Scores in Lymphoblasts Compared with Small Lymphocytes; the Figures for Small Lymphocytes were Obtained by Amalgamation of the Two Groups in Table XVI (with the Exception of G6PD for which Only the 10 Non-leukaemic Values are Stated)

	LD	α -GPD-NAD	β -HBD	MD	GD	α -GPD	SD	G6PD
Lymphoblasts	86.5 ± 34.3	25.1 ± 22.5	35.6 ± 18.9	86.3 ± 39.2	19.3 ± 14.7	8.0 ± 6.1	16.8 ± 9.1	30.4 ± 20.6
(No. of patients)	(24)	(24)	(12)	(12)	(12)	(11)	(11)	(24)
Small Lymphocytes	36.1 ± 7.3	24.3 ± 6.8	22.5 ± 5.8	37.1 ± 5.8	16.3 ± 4.8	9.6 ± 3.2	14.8 ± 4.4	26.4 ± 9.0
(No. of patients)	(20)	(20)	(20)	(20)	(20)	(20)	(20)	(10)
Significance of difference of means (P)	<0.01	>0.5	<0.05	<0.01	>0.5	>0.1	>0.1	>0.1

11.1.5 Chronic lymphocytic leukaemia lymphocytes Marrow

or blood smears were made from 17 patients with chronic lymphocytic leukaemia and the mean enzyme scores compared with those obtained for blood lymphocytes in 10 normal adults (see Table XVII). There was a significant ($P < 0.01$) increase in enzyme activity in the leukaemic lymphocyte for LD and MD only.

11.1.6 Burkitt lymphoma cell lines EB2, EB4 and Jijoye

Burkitt lymphoma cell lines maintained in long-term culture were also studied for dehydrogenase enzyme activity (see Fig. 16). The cells were diluted in normal saline, spun directly on to glass slides using a cyto-centrifuge, and the activity for 50 cells determined for each enzyme. In view of the relatively large size of these cells a larger field stop aperture was used during microdensitometry. The EB2 cell line was studied on 12 occasions and the other lines on two occasions (see Table XX). The pattern of enzyme activity was similar for the three cell lines, and similar to that for the leukaemic lymphoblast. The mean scores for EB2 cells showed a significant ($P < 0.01$) reduction in activity of enzymes of the α -glycerolphosphate shuttle (α -GPD and α -GPD-NAD) and the acetoacetate- β -hydroxybutyrate shuttle (β -HBD), but with no/

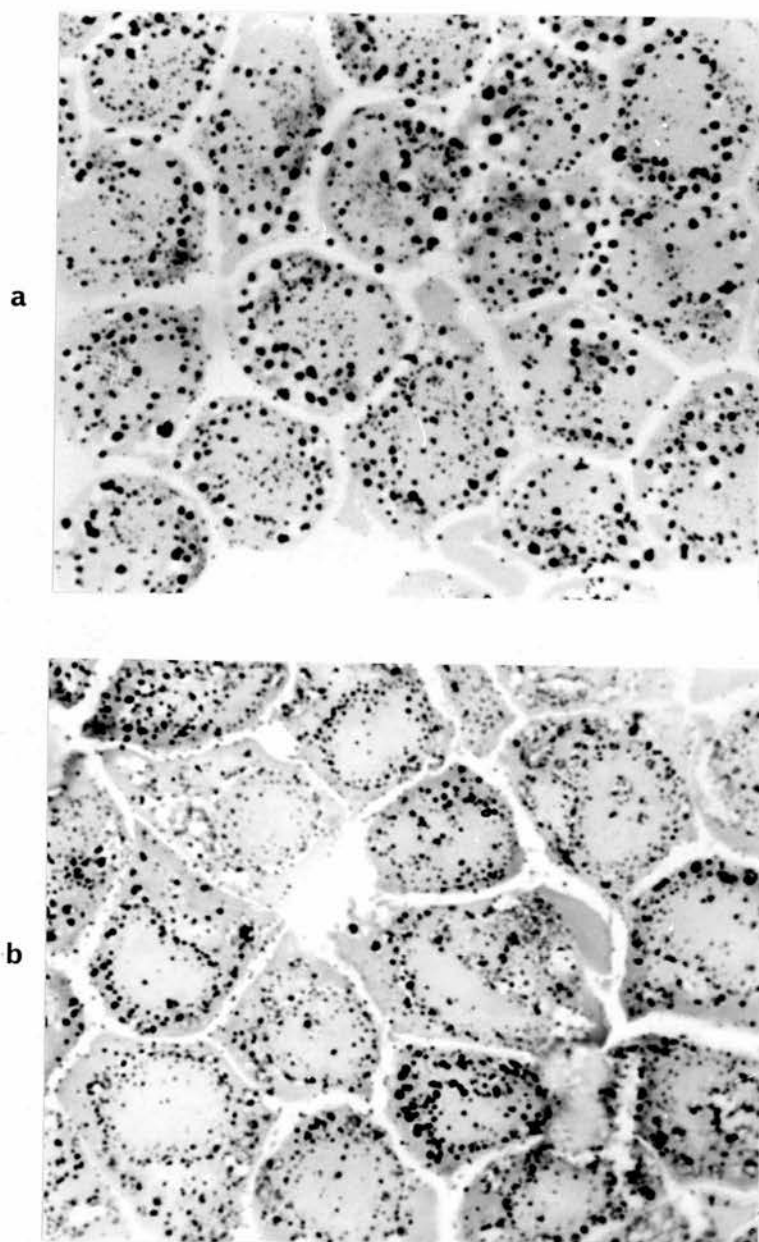


Fig. 16. Dehydrogenase enzyme activity in
EB2 cells counterstained with methyl green.
x 864. a. LD; b. MD

TABLE XX

Mean Figures (\pm SD) for Enzyme Scores in Three Burkitt Lymphoma Cell Lines and in Lymphoblasts from a Total of 40 Cases of Acute Leukaemia (Number of Patients in Parenthesis)

	LD	α -GPD-MAD	β -HBD	MD	GD	α -GPD	SD	G6PD
Burkitt lymphoma cell lines EB2 (12)	86.7	+7.2	+12.0	+75.7	+16.2	+4.0	+23.1	+28.2
	\pm 17.8	\pm 2.7	\pm 2.9	\pm 16.3	\pm 6.7	\pm 1.9	\pm 3.5	\pm 4.5
EB4 (2)	85.4	8.4	14.6	76.6	8.9	3.2	21.5	27.5
Jijoye (2)	114.6	12.2	16.2	90.9	12.0	8.6	23.3	42.5
Acute leukaemia lymphoblast	94.8	+24.2	+37.7	+91.1	+17.4	+10.3	+20.3	+33.4
	\pm 36.1 (40)	\pm 20.4 (40)	\pm 19.1 (28)	\pm 36.8 (28)	\pm 11.6 (31)	\pm 8.1 (30)	\pm 14.7 (30)	\pm 19.0 (39)
Significance of difference of means (P) Lymphoblasts v. EB2 cells	> 0.1	< 0.01	< 0.01	> 0.05	> 0.5	< 0.01	> 0.1	> 0.05

no significant ($P > 0.05$) alteration in the activity of other enzymes.

11.1.7 Effect of altering gas phase Hypoxia is known to inhibit respiration and increase glycolysis and, in order to determine whether the enzyme pattern obtained in lymphoblasts was related to oxygen availability, their enzyme activity was determined with and without exposure to hyperbaric oxygen at a pressure of 40 lb/sq in absolute for 60 min. Ten EDTA-anticoagulated bone marrow aspirates containing a high percentage of blast cells were subdivided into open containers and one portion of each incubated at 37°C in hyperbaric oxygen in a thermostatically controlled tank designed for this purpose (see Fig. 17). The remaining portions were maintained at $0-4^{\circ}\text{C}$ in air at atmospheric pressure. Smears were then made from both specimens and processed together.

This short period of exposure to hyperbaric oxygen resulted in no significant ($P > 0.1$) alteration in the activity of either intra- or extra-mitochondrial enzymes (see Table XXI).

11.2 Enzyme activity in myeloid cells

11.2.1 Normal myeloid precursors Eight normal marrows were/

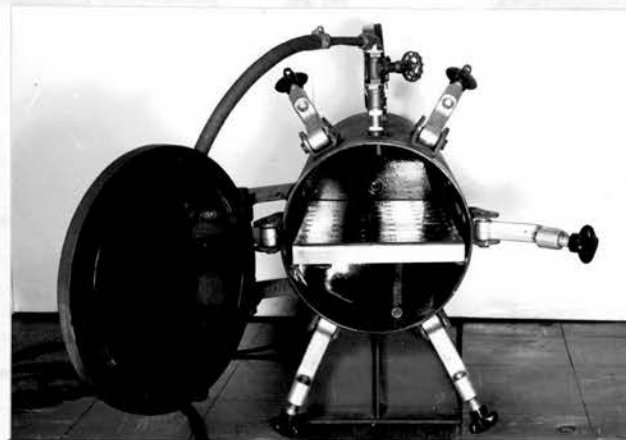
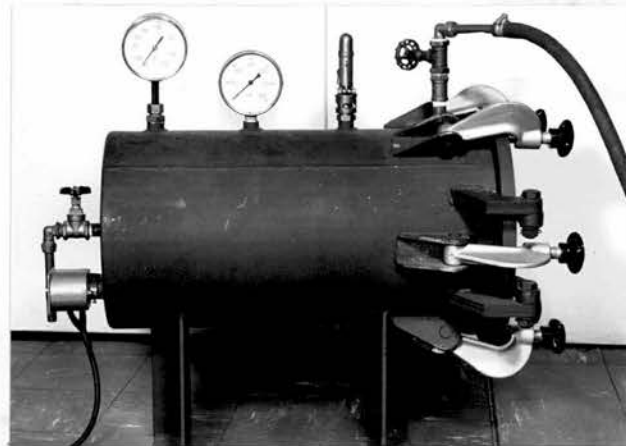


Fig. 17. Hyperbaric oxygen tank, showing pressure and temperature recording gauges, oxygen in-put tube and exit valve, and heating element with thermostat.

were studied for intra- and extra-mitochondrial enzyme activity at each of three stages of maturation: polymorph, metamyelocyte and myelocyte/promyelocyte. It was not possible to differentiate between myelocytes, promyelocytes and myeloblasts due to the limitation of the methyl green counterstain. Myelocytes and promyelocytes were therefore scored together, and since blast cells constituted < 1% of myeloid cells their contribution was ignored.

A progressive decrease in enzyme activity with increasing maturation was found for both intra- and extra-mitochondrial enzymes (see Table XXII).

11.2.2 Chronic myeloid leukaemia leucocytes A similar study of marrow and blood leucocytes was carried out in nine patients with chronic myeloid leukaemia. A progressive decrease in enzyme activity, for the same three stages of maturation, was again noted (see Table XXII).

When the normal and chronic myeloid leukaemia mean enzyme scores were compared at each of the three stages of maturation then no significant ($P > 0.05$) difference was found, with the sole exception of α -GPD ($P < 0.02$) at the myelocyte/promyelocyte stage.

11.2.3/

TABLE XXII

Mean Figures (\pm SD) for Enzyme Scores in Normal, and Chronic Leukaemic, Myeloid Cells at Three Stages of Maturation. Number of Patients in Parenthesis

	LD	α -GPD-	β -HBD	MD	GD	α -GPD	SD	GGPD
Normal marrow (8)								
1. Polymorph	+56.7 -32.8	+37.1 -18.2	+20.8 -12.4	+43.2 -18.0	+14.4 -8.2	+9.2 -8.9	+12.3 -5.5	+35.6 -16.7
2. Metamyelocyte	+76.5 -42.1	+50.9 -27.4	+31.5 -15.5	+60.5 -28.3	+23.3 -17.5	+14.1 -5.2	+17.4 -6.9	+44.5 -24.6
3. Myelo] Promyelo] cyte	+115.6 -65.3	+70.9 -48.7	+52.1 -26.7	+94.3 -51.3	+36.0 -27.3	+21.1 -7.8	+27.8 -10.4	+57.1 -37.3
Chronic myeloid leukaemia (9)								
4. Polymorph	+45.0 -8.6	+24.5 -7.7	+20.7 -2.7	+41.1 -7.8	+18.5 -5.6	+11.9 -4.4	+14.0 -3.1	+33.3 -6.5
5. Metamyelocyte	+56.7 -13.9	+39.2 -19.3	+30.4 -6.2	+52.0 -10.6	+24.3 -8.7	+17.9 -8.2	+21.0 -12.3	+43.3 -10.8
6. Myelo] Promyelo] cyte	+86.0 -21.6	+53.1 -22.1	+53.0 -15.4	+73.9 -18.1	+41.6 -11.1	+32.5 -12.8	+36.2 -11.3	+58.3 -15.5
Significance of difference of means (P)	1 v. 4 2 v. 5 3 v. 6	> 0.1 > 0.1 > 0.1	> 0.05 > 0.1 > 0.1	> 0.5 > 0.5 > 0.5	> 0.5 > 0.1 > 0.5	> 0.1 > 0.5 > 0.5	< 0.02 > 0.1 > 0.1	> 0.5 > 0.5 > 0.5

11.2.3 Leukaemic myeloblasts and monoblasts Six cases of pure myeloblastic leukaemia (confirmed by the Sudan Black reaction and excluding promyelocytic, monocytic and myelo-monocytic leukaemias) with scanty promyelocytes and myelocytes in the marrow were compared with six cases of monocytic or myelo-monocytic leukaemia (confirmed by the presence of diffuse scattered granules of Sudan Black positivity). Fifty blast cells were scored in each marrow and the results are given in Table XXIII. Myeloblasts showed a significant ($P < 0.05$) reduction in activity of the intra-mitochondrial enzymes α -GPD and SD, the α -glycerolphosphate shuttle enzyme α -GPD-NAD, and the pentose phosphate shunt enzyme G6PD, compared with the blast cells in myelo-monocytic leukaemia.

The mean enzyme scores for myeloblasts were compared with the mean scores previously obtained for myelocytes/promyelocytes in the eight normal marrows. No significant ($P > 0.1$) difference in enzyme activity was found.

12. Discussion

The object of these experiments was to study the activity, relative to one another, of the intra- and extra-mitochondrial dehydrogenases and to assess the activity of/

TABLE XXIII

Mean Figures (\pm SD) for Enzyme Scores in Acute Myeloblastic and Monocytic/Myelo-monocytic Leukaemias. Number of Patients in Parenthesis.

	LD	α -GPD- NAD	β -HBD	MD	GD	α -GPD	SD	G6PD
Acute leukaemia blast cells								
1. Myeloblastic (6)	± 131.9 -20.1	± 48.5 -24.3	± 71.2 -19.8	± 112.3 -20.7	± 37.6 -11.9	± 16.6 -6.2	± 29.0 -5.8	± 35.7 -12.2
2. Monocytic and myelo-mono- cytic (6)	± 153.3 -38.7	± 80.2 -18.5	± 67.2 -12.2	± 135.2 -24.0	± 49.9 -24.5	± 46.8 -24.7	± 62.8 -21.7	± 96.9 -56.1
Significance of difference of means (P)								
1 v. 2	> 0.1	< 0.05	> 0.5	> 0.1	> 0.1	< 0.05	< 0.01	< 0.05
1 v. 3	> 0.5	> 0.1	> 0.1	> 0.1	> 0.1	> 0.1	> 0.5	> 0.1
(For 3 see Table XXII)								

of mitochondrial membrane shuttle systems in leucocytes at different stages of maturation. Boxer (1965) observed that the activity of a large number of extra-mitochondrial glycolytic enzymes maintained a constant proportion one to another in both normal and malignant tissues; an exception to this was a selective decrease in activity of the α -glycerolphosphate shuttle enzyme α -GPD-NAD in malignant cells. In this study of leucocyte maturation a fairly constant proportion was always maintained between glycolytic and respiratory enzyme activity with the exception of leukaemic lymphoblasts and Burkitt lymphoma EB2 cells. In these two exceptions there was a disproportionately low activity of the intra-mitochondrial enzymes, and of α -GPD-NAD, with a contrasting high extra-mitochondrial glycolytic enzyme activity. There was also evidence of a low activity of the acetoacetate- β -hydroxybutyrate shuttle enzyme β -HBD in EB2 cells but not of the malate shuttle enzyme MD in any of the cells studied.

In developing leucocytes the activity of α -GPD-NAD appeared to be directly proportional to intra-mitochondrial enzyme activity, and this would suggest that when mitochondrial enzyme activity is low then little hydrogen traverses the α -glycerolphosphate shuttle. In the lymphoblast/

lymphoblast and EB2 cell the relatively high activity of LD suggests that hydrogen may instead be taken up in the extra-mitochondrial reduction of pyruvate to lactate. Both of these cell types may therefore be predominantly dependent on glycolysis, and the continuing activity of LD, for energy requirements.

The relatively low respiratory enzyme activity in these cells could, in theory, be due to 1. hypoxia leading to respiratory depression, 2. differences in cell proliferative rate, 3. a direct stimulus to glycolysis and 4. the presence of scanty mitochondria.

The failure of hyperbaric oxygen to increase mitochondrial and α -glycerolphosphate shuttle enzyme activity in lymphoblasts suggests that their enzyme pattern is not an immediate consequence of oxygen deprivation. It is also unlikely that this enzyme pattern is due to a difference in cell proliferative rate since the majority of lymphoblasts show a low mitotic activity (Mauer and Fisher, 1962; Gavosto, Pileri, Bachi and Pegoraro, 1964; Killman, 1965; Foadi, Cooper and Hardisty, 1968) whereas Burkitt lymphoma cells show a relatively high mitotic activity (Epstein and Barr, 1965; Cooper, Hughes and Topping, 1966). It is not possible to determine from these studies whether a glycolytic enzyme stimulant/

stimulant exists in lymphoblasts and EB2 cells although this seems a remote possibility. There is, however, evidence for a relationship between mitochondrial number and the activity of intra-mitochondrial and α -glycerol-phosphate shuttle enzymes.

A progressive decrease in both mitochondrial and extra-mitochondrial enzyme activity with maturation was observed in normal myeloid cells and in normoblasts and megaloblasts, and is in keeping with previous cytochemical studies of a smaller number of enzymes (Hayhoe, 1963; Ackerman, 1964; Hayhoe et al., 1964; Rozenszajn and Shoham, 1967). The decrease in mitochondrial enzyme activity is probably related to mitochondrial number since developing cells of the myeloid series (Pease, 1956; Bessis, 1956; Kakefuda, 1968) and erythroid series (Wintrobe, 1967) show a parallel decrease in the number of mitochondria with differentiation.

Cells of the lymphoid series, in contrast, contain scanty mitochondria at all stages of maturation - including the lymphoblast (Kakefuda, 1968), the Burkitt lymphoma cell (Epstein and Achong, 1970) and the small lymphocyte (Kakefuda, 1968). Thus, as the lymphoblast develops into the small lymphocyte there is a substantial decrease in extra-mitochondrial/

extra-mitochondrial glycolytic enzyme activity whereas mitochondrial and shuttle system enzymes show low activity at all stages. This suggests that if precursor cells have a higher metabolic requirement for ATP, for proliferative activity, then the synthesis of ATP will predominantly depend on glycolysis.

The chronic lymphocytic leukaemia lymphocyte also showed low mitochondrial and shuttle system enzyme activity and is known to contain scanty mitochondria (Kakefuda, 1968; Schrek, Mayron and Knospe, 1971) in approximately equal number to the non-leukaemic lymphocyte. The increased activity of extra-mitochondrial LD and MD in chronic lymphocytic leukaemia does suggest a similar pattern to the high glycolytic enzyme activity of the lymphoblast and this increase was more obvious in those cases of lymphocytic leukaemia in which the lymphocytes were predominantly of the large cell type.

Under certain conditions, however, the lymphocyte can increase its mitochondrial number - in the PHA-transformed cell (Inman and Cooper, 1965; Cooper, 1969) and in the virus-induced atypical mononuclear of infectious mononucleosis (Paegle, 1961; Inman and Cooper, 1965). In the latter, however, the increase is probably related to the predominance of a 'lymphoid' or 'monocytoid' type of/

of atypical mononuclear. No significant increase in enzyme activity was found for either intra- or extra-mitochondrial enzymes in atypical mononuclears in these experiments.

A significant increase in activity of all enzymes was, however, seen in the PHA-transformed cell at 72 hours. Gough and Elves (1967) reported similar results for a smaller number of enzymes. The increase in glycolytic enzyme activity would be expected in view of the dependence of these cells on glycolysis for transformation (Nowell, 1960; Cooper, Barkhan and Hale, 1963; Parenti, Franceschini, Forti and Cepellini, 1966; Polgar, Foster and Cooperband, 1968) and the associated increase in mitochondrial and shuttle system enzymes is presumably a consequence of the increase in mitochondrial number. This is an important example since it reinforces the correlation of mitochondrial enzyme activity with mitochondrial number rather than with metabolic changes which temporarily alter the relative activity of the respiratory and glycolytic pathways.

The difference in enzyme activity between the blast cells of acute myeloblastic and myelo-monocytic leukaemia may also be explained on the basis of mitochondrial number. The increase in mitochondrial and shuttle system enzyme activity in blast cells showing monocytoid characteristics parallels an increase in mitochondrial/

mitochondrial number in these cells compared with myeloblasts (Freeman and Journey, 1971). Myeloblasts also contain more numerous mitochondria than lymphoblasts and this was also associated with an increase in their enzyme activity.

These results for leukaemic blast cells are not, therefore, in keeping with previous reports of a selective deletion of α -GPD-NAD in neoplastic cells of solid tumours. The relative low activity of this enzyme in immature lymphoid cells was not selective, since a decrease in intra-mitochondrial enzyme activity was invariably associated with it. Since low α -GPD-NAD activity was not a characteristic of all types of leukaemic blast cells these enzyme changes appear to be related to cell line and mitochondrial number.

In solid tumours, the reduction in activity of α -GPD-NAD may also, in some cases at least, be related to mitochondrial number since, in neoplastic cells, the number of mitochondria is usually smaller than in normal cells from the same tissue of origin (Wenner, Spirtes and Weinhouse, 1951; Wenner and Weinhouse, 1953; Schneider, Hogeboom, Shelton and Striebich, 1953; Kit and Griffin, 1958; Aisenberg, 1961). In general, the number of mitochondria in a tissue correlates more with its/

its functional activity than with its mitotic activity, and actively functioning specialised cells usually show active respiration compared with rapidly dividing less differentiated tissues which require only a limited respiratory apparatus (Aisenberg, 1961). Thus, future studies of glycolytic, respiratory and mitochondrial membrane shuttle system enzymes in solid tumours should relate enzyme activity to mitochondrial number and not merely to tissue nitrogen content or wet weight. It would thus seem essential to obtain a reference normal control tissue with approximately the same number of mitochondria as the malignant tissue to allow a valid comparison in tissue homogenate studies to be made. A failure to take this factor into account would also provide spurious support for the Warburg hypothesis.

**PART V: PENTOSE PHOSPHATE SHUNT, TRANSHYDROGENASE
AND DIAPHORASE ENZYME ACTIVITY IN LEUKAEMIC LEUCOCYTES**

1. Pentose Phosphate Shunt Enzyme Activity

1.1 Introduction The significance of the pentose phosphate shunt in cell metabolism is two-fold: first, it results in the generation of the pentose sugars of ribose and deoxyribose which are required for nucleoprotein synthesis; secondly, it allows the generation of NADPH_2 which is used in a number of biosynthetic reactions (see Fig. 18) including tetrahydrofolate synthesis (Futterman, 1957); steroid synthesis (Popják, 1961; Mitton and Boyd, 1967); and fatty acid synthesis (Kornberg, 1961). If the requirement for NADPH_2 in biosynthesis is low (e.g. in a tissue which is not rapidly proliferating) the hydrogen from NADPH_2 will follow the microsomal cytochrome P450, or diaphorase, system (Smuckler, Arrhenius and Hultin, 1967; Slater, 1968) to oxygen, without being channelled into NADPH_2 -dependent processes; or it may instead be directed by a trans-hydrogenation mechanism to form NADH_2 for intra-mitochondrial ATP synthesis (Colowick, Kaplan, Neufeld and Ciotti, 1952; Kaplan, Colowick and Neufeld, 1953). In rapidly proliferating tissues, however, most of the hydrogen of NADPH_2 will be used for biosynthesis and will not pass along the diaphorase-cytochrome system.

Because of the requirements of rapidly proliferating tissues/

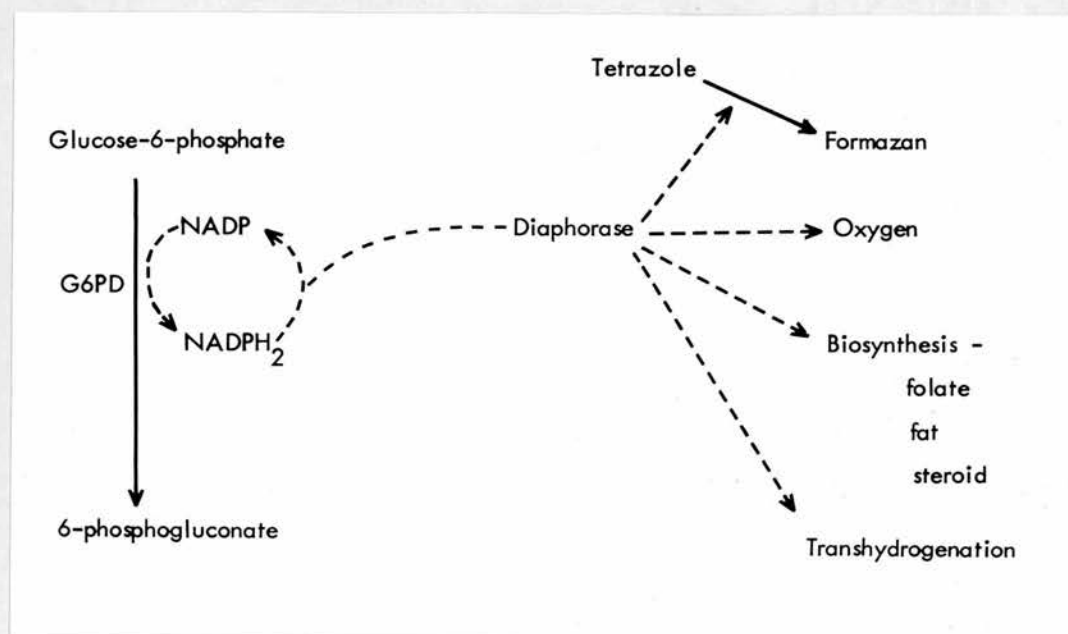


Fig. 18. Potential routes of reoxidation of NADPH₂.

tissues for pentose phosphate shunt generated pentose sugars and NADPH_2 , the activity of its dehydrogenating enzymes G6PD and 6PGD have been extensively studied in malignant tissues. Many conflicting reports have been published: Chayen, Bitensky, Aves, Jones, Silcox and Cunningham (1962) found increased 6PGD activity, while Boxer (1965) found no significant increase, in malignant tissue; Harcourt-Webster and Stott (1966) reported decreased G6PD activity in anaplastic tumours while Thiery and Willighagen (1965) found the converse; more recently, Smith, King, Meggitt and Allen (1970) reported increased G6PD activity in breast carcinoma. These, and other, conflicting reports presumably reflect differences in cell proliferative rate and degree of differentiation in solid tumours.

In leukaemia, conflicting results have also been obtained. Beck (1958a) found a decrease in activity in the chronic leukaemias, which appeared to be related to a decrease in hexokinase activity, while Harrap and Jackson (1969) reported increased G6PD activity in chronic myeloid leukaemia. Seitz and Luganova (1968) reported decreased activity in acute and chronic lymphatic leukaemia and in chronic myeloid leukaemia, Bertino, Alenty, Gabrio and Huennekens (1960) found decreased activity in the leukaemic lymphoblast/

lymphoblast, and Brody and Merlie (1970) found decreased activity in the chronic leukaemic lymphocyte. Hayhoe et al. (1964), using a cytochemical technique, obtained variable results in blast cells which appeared to be inversely related to P.A.S. positivity.

The enzyme G6PD, as representative of pentose phosphate shunt activity, was therefore studied in normal and leukaemic leucocytes at varying stages of maturation in order to determine whether any significant alteration in activity could be related to leukaemic change.

1.2 Methods, patients studied and results The method used has already been described (see IV.6) and the values for G6PD activity have already been stated in the tables of intra- and extra-mitochondrial enzyme activity (see Tables XVI to XXIII).

P.A.S. positivity was assessed using the method and semi-quantitative scoring technique of Hayhoe et al. (1964).

No significant increase in G6PD activity was found in the lymphoblast compared with the small lymphocyte ($P > 0.1$); in the myeloblast compared with the myelocyte/promyelocyte ($P > 0.1$); in the chronic lymphocytic leukaemia lymphocyte compared with the small lymphocyte ($P > 0.05$)/

($P > 0.05$); or in the atypical mononuclear compared with the small lymphocyte ($P > 0.5$). G6PD activity decreased with maturation in both normal myeloid and erythroid cells and this pattern was also observed in the myeloid hyperplasia of chronic myeloid leukaemia and the maturation arrest of megaloblastic erythropoiesis.

The relationship between P.A.S. positivity and the relative activities of LD, α -GPD-NAD, SD, G6PD and c-NAD is shown in Figs. 19 and 20.

1.3 Discussion G6PD is a representative enzyme of the pentose phosphate shunt but it is difficult to correlate demonstrable G6PD activity with individual metabolic functions of this pathway. The hydrogen generated by this dehydrogenase reaction may be utilised for nucleoprotein synthesis, or fat, steroid or folate biosynthesis; or may, alternatively, be passed via a transhydrogenase reaction to the pathways involved in energy production (see Fig. 18). Thus the relatively normal G6PD activity of blast cells in this study may mask a redeployment of reducing equivalents away from nucleoprotein synthesis, as a consequence of low mitotic activity, towards alternative pathways.

G6PD is not an enzyme connected with glycogen biosynthesis but, as a rule, its activity decreases in parallel/

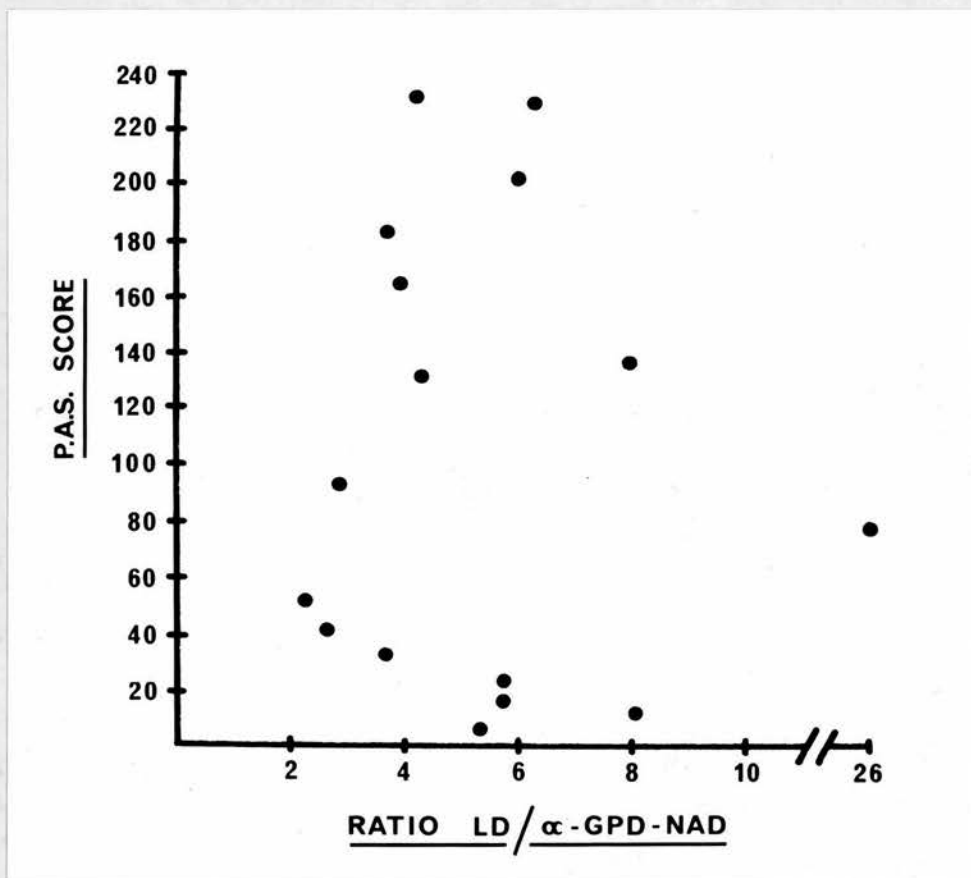


Fig. 19. Relationship between P.A.S. score and the enzyme activity ratio LD/α-GPD-NAD in lymphoblasts in 16 cases of acute leukaemia.

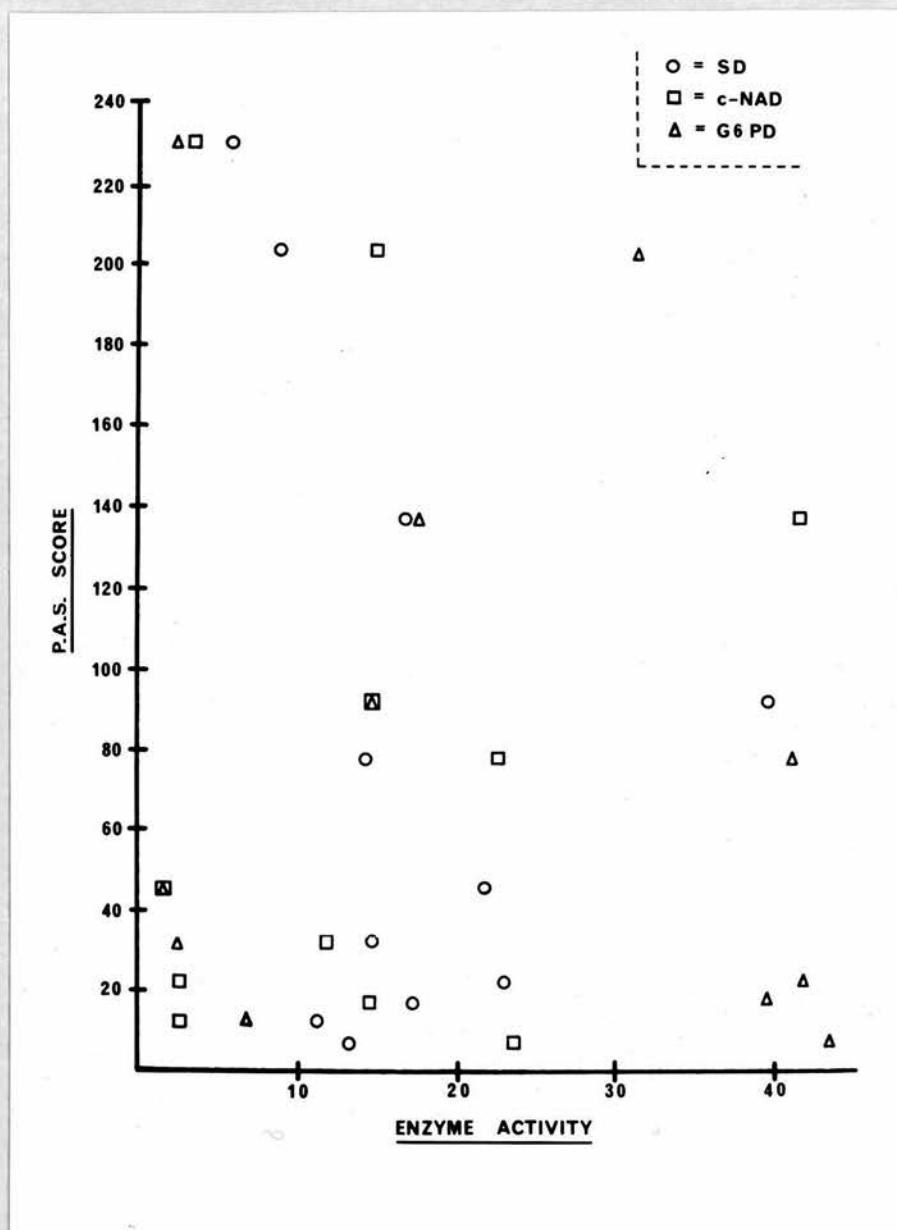


Fig. 20. Relationship between P.A.S. score and the activity of SD, G6PD and c-NAD in lymphoblasts in 11 cases of acute leukaemia.

parallel with a decrease in activity of the enzymes of the glycogen synthetic pathway (Seitz, 1965). The P.A.S.-reactive material in leucocytes consists almost entirely of glycogen (Gahrton, 1966) and Hayhoe et al. (1964) suggested that an apparent inverse relationship between G6PD activity and P.A.S. positivity might reflect decreased utilization of carbohydrate as a consequence of depression of pentose phosphate shunt activity.

This correlation did not hold true for the larger number of cases in this study and there was also no correlation between blast cell glycogen content and mitochondrial enzyme activity (SD), the ratio LD : α -GPD-NAD, or endogenous (c-NAD) activity. Thus it was not possible to draw any conclusion with regard to blast cell immaturity (as judged by glycogen depletion: Seitz, 1965) and the characteristic LD : α -GPD-NAD imbalance, or mitochondrial enzyme activity.

2. Pyridine Nucleotide Transhydrogenase Activity

2.1 Introduction Co-factor concentrations are of importance in the regulation of glycolysis and respiration. The amount of NAD available for the Embden-Meyerhof pathway in mammalian cells is small (Kaplan, 1960) and the pathway would soon cease to operate without mechanisms for the reoxidation of NADH_2 to NAD (Staunton West et al., 1966).

In/

In the pentose phosphate pathway in leucocytes NADP is again not present in great abundance, although the level is probably sufficiently adequate not to be primarily rate-limiting (Beck, 1958b), and factors which facilitate the oxidation of NADPH_2 are able to further stimulate the activity of the shunt (Iyer, Islam and Quastel, 1961; Evans and Karnovsky, 1962). Thus the ratios of $\text{NAD}:\text{NADH}_2$ and $\text{NADP}:\text{NADPH}_2$, or the redox state of these pyridine nucleotides (Krebs and Veech, 1969), is important in the regulation of cell metabolism.

Colowick et al. (1952) and Kaplan, Colowick and Neufeld (1953) described a pyridine nucleotide transhydrogenase which catalysed the reaction: $\text{NADPH}_2 + \text{NAD}^+ \rightleftharpoons \text{NADP} + \text{NADH}_2$. In 1963, Silber, Huennekens and Gabrio described this transhydrogenase in normal and leukaemic leucocytes and suggested that it might play an important role in the regulation of reduced pyridine nucleotide co-enzymes. A cytochemical method was therefore devised to demonstrate the activity of this transhydrogenase in normal and leukaemic leucocytes.

2.2 Methods The unfixed smear technique incorporating a 40% w/v concentration of Ficoll in the incubation solution was used and formazan production was assessed by/

by microdensitometry. A final concentration of 1 mg/ml of NADPH_2 was used as substrate and the standard concentrations of NBT and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ were added to glycylglycine buffer (see Table XXIV). When 1 mg/ml of NAD was added to this reaction mixture there was a considerable increase in formazan production; this gross increase was greater than the effect of adding 1 mg/ml of NAD to the control reaction (c) obtained in the absence of NADPH_2 . The net increase due to the effect of NAD added to NADPH_2 was therefore obtained by subtracting the value obtained for the effect of NAD added to the control reaction (see Table XXV). This net increase in formazan production was assumed to arise from the generation of NADH_2 , thus representing a transhydrogenation effect (Butcher and Chayen, 1968).

2.3 Patients studied and results The mean figure for 50 blast cells in each of 10 patients with acute lymphoblastic leukaemia was compared with the mean figures obtained for marrow lymphocytes in 12 children who did not suffer from leukaemia. A significant ($P < 0.02$) increase in transhydrogenase activity for blast cells was found (see Table XXV).

The transhydrogenation effect arising from PHA-transformation of small lymphocytes was determined in the same/

TABLE XXIV

Composition of Incubation Solutions for the
Demonstration of Transhydrogenase Enzyme Activity

	NADPH ₂	NADPH ₂ + NAD	c	c-NAD
Substrate (mg)	5	5	-	-
Buffer (ml)	5	5	5	5
NBT (mg)	5	5	5	5
CaCl ₂ ·2H ₂ O (mg)	65	65	65	65
Ficoll (g)	2	2	2	2
NAD (mg)	-	5	-	5

TABLE XXV

Mean Figures (\pm SD) for Transhydrogenation Effect in Marrow Lymphoblasts and Small Lymphocytes; and in PHA-transformed Peripheral Blood Lymphocytes

	NADPH ₂	NADPH ₂ +NAD	INCREASE DUE TO NAD (GROSS)	c	c-NAD	INCREASE DUE TO NAD	TRANS- HYDROGENATION EFFECT DUE TO NAD (NET)	SIGNIFICANCE OF DIFFERENCE OF MEANS (P)
Lymphoblasts (10 marrow)	+63.5 -27.6	+107.5 -42.9	+44.0 -23.2	+6.8 -7.6	+15.0 -11.5	+8.2 -6.6	+35.8 -20.8	< 0.02
Small lympho- cytes (12 marrow)	+39.3 -14.1	+60.3 -22.2	+21.0 -10.7	+3.8 -3.9	+7.3 -6.4	+3.5 -3.3	+17.5 -8.3	
PHA-transformed lymphocytes (72 hr culture)	+90.0 -14.3	+126.2 -11.4	+36.2 -18.5	+11.2 -3.9	+17.9 -3.6	+6.7 -3.2	+29.5 -18.6	< 0.05
Small lympho- cytes (1 hr culture)	+34.2 -2.9	+48.0 -2.8	+13.7 -2.8	+7.3 -0.9	+10.1 -2.3	+2.8 -2.0	+10.9 -1.5	

same way by comparing the enzyme activity after one hour in 199 culture medium without added PHA, with the enzyme activity obtained after culture for 72 hours in the presence of PHA. A significant ($P < 0.05$) increase in activity in the transformed cells was obtained for a total of five transformations (see Table XXV).

2.4 Discussion The increase in transhydrogenation in PHA-transformed lymphocytes would appear to parallel the general increased metabolic activity of these cells which is illustrated by an increase in the activity of the pentose shunt, Embden-Meyerhof pathway and tricarboxylic acid cycle enzymes. The increased transhydrogenase activity in leukaemic blast cells has previously been reported in tissue homogenate studies (Evans and Kaplan, 1966; Evans and Getz, 1968). G6PD, and therefore pentose phosphate shunt, activity was, however, not increased in the lymphoblast and this may be a consequence of the relatively low mitotic rate and requirement for nucleoprotein synthesis in these cells (Mauer and Fisher, 1962; Gavosto et al., 1964; Killmann, 1965; Foadi et al., 1968).

Thus the increase in transhydrogenase activity may represent a redeployment of reducing equivalents from $\text{NADPH}_2/$

NADPH_2 to NADH_2 to be utilised, for example, in the tricarboxylic acid respiratory cycle. In the same patients the relatively low α -glycerolphosphate and respiratory pathway enzyme activity in blast cells also leads to NADH_2 accumulation, and there may, therefore, be considerable metabolic dependence on LD in order to achieve reoxidation of NADH_2 via the pyruvate-lactate interaction.

3. Diaphorase Enzyme Activity

3.1 Introduction When NADPH_2 and NADH_2 are generated as the result of a dehydrogenase reaction the reduced co-enzymes become, in turn, the substrate for enzyme systems which oxidise the co-enzyme and pass the hydrogen to the cytochrome system (see Fig. 18). In the presence of a tetrazolium salt, the hydrogen is used instead to reduce the tetrazole and generate formazan. This enzyme (diaphorase) system simultaneously allows NADP and NAD to be regenerated, and the two diaphorases which interact with those co-enzymes are known as NADPH_2 -diaphorase and NADH_2 -diaphorase respectively.

Unless this diaphorase system is by-passed (by the use of an additional hydrogen acceptor such as phenazine methosulphate) the formazan generated in the presence of a tetrazole reflects the combined activity of the dehydrogenase/

dehydrogenase and its respective diaphorase (see Fig. 18). It is important to determine, therefore, that diaphorase activity is not lower than dehydrogenase activity in a cell or tissue since it would then become rate-limiting. The activity of NADPH_2 and NADH_2 diaphorases was therefore determined cytochemically in normal marrow lymphocytes and in leukaemic lymphoblasts.

3.2 Methods A similar technique to the method for dehydrogenase enzyme determination, but incorporating a 1 mg/ml concentration of NADPH_2 or NADH_2 as substrate (see Table XXVI), was used. Formazan production was measured by microdensitometry in 25-50 cells for each marrow studied and the control values for c-NADP and c-NAD subtracted to give net enzyme activity. The strongest reacting NADP- and NAD-dependent dehydrogenases (G6PD and LD respectively) were also estimated in the same patients.

3.3 Patients studied and results Diaphorase enzyme activity was measured in lymphoblasts in 15 cases of acute leukaemia and in marrow small lymphocytes in 12 non-leukaemic children (see Table XXVII). The mean scores for diaphorase activity were higher for lymphoblasts than for small lymphocytes. In the small lymphocyte, the mean scores obtained for each of the two diaphorases was significantly/

TABLE XXVI

Composition of Incubation Solutions for the
Demonstration of NADPH₂ and NADH₂ Diaphorase Activity

	NADPH ₂ -DIAPHORASE	NADH ₂ -DIAPHORASE
Buffer (ml)	5	5
NBT (mg)	5	5
CaCl ₂ ·2H ₂ O (mg)	65	65
Ficoll (g)	2	2
NADPH ₂ (mg)	5	-
NADH ₂ (mg)	-	5

TABLE XXVII

Mean Figures (\pm SD) for Diaphorase and Dehydrogenase Enzyme Activity in Marrow Lymphocytes and Lymphoblasts. Number of Patients Studied in Parenthesis

	NADPH ₂ -DIAPHORASE	G6PD	NADH ₂ -DIAPHORASE	LD
Small Lymphocyte (12)	39.8 ± 12.0	24.2 ± 10.6	56.8 ± 13.1	36.7 ± 5.6
Significance of difference of means (P)	< 0.01		< 0.01	
Lymphoblast (15)	54.3 ± 28.0	30.5 ± 22.6	99.8 ± 48.1	82.4 ± 39.1
Significance of difference of means (P)	< 0.02		> 0.1	

significantly higher ($P < 0.01$) than for the corresponding strongest reacting dehydrogenase. In the lymphoblast, the activity of NADPH_2 -diaphorase was also significantly higher ($P < 0.02$) than the G6PD activity, but the activity of NADH_2 , although greater than that of LD, was not significantly increased ($P > 0.1$).

3.4 Discussion Altmann, Bitensky, Butcher and Chayen (1970), in a histochemical study of experimentally induced rat hepatoma, reported decreased activity of G6PD compared with normal liver. This was believed to be secondary to a decrease in the activity of NADPH_2 -diaphorase, and an alternative hydrogen-accepting system, employing glutathione and ascorbate, was postulated. In the leukaemic lymphoblast, compared with the small lymphocyte, there is no reduction in G6PD activity and there is an increase in NADPH_2 -diaphorase activity. Thus the diaphorase is not rate-limiting in respect of pentose phosphate shunt enzyme activity and, to the contrary, there appears to be potential for additional hydrogen transport via this pathway.

The net activity for LD in the lymphoblast is, however, close to the maximum activity of the NADH_2 -diaphorase. This is consistent with the increased glycolytic enzyme activity of these cells and this may be secondary to the relatively/

relatively low respiratory enzyme activity and to the increased transhydrogenation from NADPH_2 .

**PART VI: POTENTIAL CYTOTOXIC EFFECT OF
GLYCOLYTIC ENZYME INHIBITORS**

1. Introduction

Inhibition of LD activity has been proposed as a potential mode of chemotherapy in the treatment of cancer, primarily because an increase in lactic acid formation is a characteristic feature of many malignant cells (Busch and Nair, 1957; Papaconstantinou, Goldberg and Colowick, 1963).

The relatively high glycolytic enzyme (LD) activity of the leukaemic lymphoblast and Burkitt lymphoma cell, in contrast to their low respiratory enzyme activity, together with reports of systemic lactic acidosis in acute leukaemia (Field et al., 1966; Rothe and Porte, 1970) and in Burkitt's lymphoma (Block, Bronson and Bell, 1966) suggest that LD inhibition might result in a cytotoxic effect in these diseases.

The selective inhibition of glycolysis, without concomitant respiratory pathway suppression, can only be achieved by inhibition of LD since this is the sole enzyme of the Embden-Meyerhof pathway which plays no role in the oxidation of carbohydrate via pyruvic acid and the tricarboxylic acid cycle (see Fig.2). Oxamic acid is a structural analogue of pyruvic acid which exhibits marked specificity as an inhibitor of LD (Hakala, Glaid and Schwert, 1953); this prevents glycolytic resynthesis/

resynthesis of ATP under both aerobic and anaerobic conditions. Under anaerobic conditions, there is reversal of oxamate inhibition of LD with time due to the accumulation of pyruvic acid (Papaconstantinou and Colowick, 1961a); in the presence of oxygen, however, pyruvate does not accumulate since it is metabolised by the mitochondrial respiratory pathway which is not significantly suppressed by oxamate (Papaconstantinou and Colowick, 1961a).

Oxamate is able to penetrate intact cells (Goldberg, 1961), inhibit LD activity in Ehrlich ascites tumour cells (Papaconstantinou and Colowick, 1961a) and in HeLa cells, and also prevent growth of the latter in culture (Papaconstantinou and Colowick, 1961b; Goldberg and Colowick, 1965).

Leukaemic lymphoblasts show low activity of intra-mitochondrial and α -glycerolphosphate shuttle system enzymes and since the NADH_2 generated in the Embden-Meyerhof pathway requires an alternative route for re-oxidation it is likely that the reduction of pyruvate to lactate fulfils this role. If this represents a metabolic dependence on glycolysis, then glycolytic enzyme inhibition will result in depleted cellular ATP levels and have a profound effect on the metabolic activity of the leukaemic/

leukaemic cell since the energy requirement for mitosis is largely dependent on glycolysis (Bullough, 1952; Polgar, Foster and Cooperband, 1968). Johnstone and Scholefield (1965) have also shown that ATP depletion can reduce cellular incorporation of amino acid into protein.

This potential cytotoxic effect would to some extent be selective for lymphoblasts under aerobic conditions if the higher respiratory activity of normal myeloid cells indicates that these cells can use the intra-mitochondrial respiratory pathway as an alternative source of ATP synthesis. It is hoped that, in the lymphoblast, this pathway is an inadequate alternative route for NADH_2 oxidation, as Herzenberg and Roosa (1960) have shown for lymphoma cells, although there may be sufficient respiratory activity to prevent the pyruvate accumulation and reversal of the inhibitory action of oxamic acid which occurs under anaerobic conditions. Since it was not possible to assess the relative metabolic dependence of normal and leukaemic cells on these pathways by cytochemical techniques, an in vitro cell culture technique was established to determine the cytotoxic effect of oxamic acid in a 48-hour maintenance culture system.

2. Methods

2.1 Cell separation technique

2.1.1 EB2 cells A long-term stock culture comprising 500,000-1,000,000 EB2 cells per ml of 199 or Eagle's culture medium (Burroughs Wellcome) containing 25% v/v foetal bovine serum was maintained by subculture at 2-3 day intervals. For cytotoxicity experiments this stock cell suspension was concentrated by centrifugation at 1,500 rpm for 10 min, resuspended in foetal bovine serum and the viable cell count determined using 0.05% trypan blue. An appropriate dilution was made in 0.5 ml foetal bovine serum, then diluted to 2 ml with 199 culture medium to give a final viable count of approximately 2,000 cells/cu mm.

2.1.2 Bone marrow cells Marrow cells were aspirated into a sterile, conical-bottomed, universal containing 1 ml of 6% dextran and 100 units of heparin (mucous). After sedimentation for 20 min at room temperature, the supernatant white cell rich plasma was removed and recentrifuged at 1,500 rpm for 6 min. The resulting cell button was washed once by resuspension in 5 ml of 0.9% saline then recentrifuged. The final cell button was resuspended in foetal bovine serum and 199 culture medium, as before, to give/

give a final viable count of approximately 2,000 cells/cu mm.

2.1.3 Blood neutrophils and lymphocytes Normal adult venous blood specimens (20 ml) were taken into two 20 ml conical-bottomed universals containing 10 ml of 3% heparin-dextran in 0.9% saline and allowed to sediment for 20 min at room temperature. The supernatant leucocyte-rich layers were removed and centrifuged at 850 rpm for 6 min to give a cell button; the resulting supernatants were recentrifuged at 3,000 rpm for 10 min to remove the platelets. The cell buttons were then resuspended in 1 ml of the platelet-poor plasma-dextran mixture, amalgamated, and layered carefully on to 5 ml of a Ficoll-Triosil mixture, as described by Harris and Ukaejiofo (1970). After centrifugation at 1,500 rpm for 20 min the lymphocytes were recovered from the white layer immediately below the Ficoll-Triosil interface, washed once in 0.9% saline, resuspended in foetal bovine serum and the viable count determined. The neutrophils were recovered from the cell button after red cell lysis, washed once in 0.9% saline and also resuspended in foetal bovine serum. A final concentration of approximately 2,000 viable cells/cu mm of 199 culture medium was made to a total volume of 2 ml.

2.2/

2.2 Cell culture technique

2.2.1 Culture medium A 20 ml volume of concentrated 199 culture medium (Burroughs Wellcome) was diluted with 173 ml sterile distilled water and 5 ml of 5% sodium bicarbonate. Two ml of a penicillin/streptomycin mixture (giving final concentrations of 100 and 74.5 international units/ml respectively) were added, and this working solution stored at 0-4°C until required. Where necessary, the final pH was adjusted to 7.2 prior to use by the addition of 5% sodium bicarbonate.

2.2.2 Oxamate and control solutions Sodium oxamate (17.8 mg) was added to 1.5 ml of 199 working solution; this provided a final oxamate concentration of 0.08M after the addition of 0.5 ml of cells suspended in foetal bovine serum, and gave an osmolality of 415 m osmol/kg (as measured by freezing point depression using an Advanced Instruments, model 31 LA osmometer). A second oxamate incubation solution of concentration 0.08M, but of osmolality 291 m osmol/kg, was prepared by prior dilution of 199 culture medium to one-third strength with sterile distilled water.

Control sodium chloride incubation solutions (0.08M) were prepared in the same way by the addition of 9.5 mg NaCl to/

to 1.5 ml full strength and one-third strength 199, to give osmolality readings of 429 and 295 m osmol/kg respectively.

2.2.3 Incubation procedure All cell cultures were established at an initial viable count of approximately 2,000 cells/cu mm in 2 ml of culture medium, at a depth of 5 mm, in a 20 ml glass siliconised universal container. A neutral pH was maintained by twice daily gassing with 5% CO₂ in air. Viable cell counts were performed at 0, 24, 48 and occasionally at 6 hours by dilution in 0.05% trypan blue in 0.9% saline using a white-cell counting pipette. A 500-cell differential leucocyte count was performed on the marrow and blood specimens by cyto-centrifugation of the remaining cells and staining by May-Grünwald Giemsa and the viable cell count was then adjusted according to the differential count.

2.3 Neutrophil phagocytosis The method was based on that of Mandell, Rubin and Hook (1970). Neutrophil concentrates were obtained from normal adult venous blood by dextran sedimentation, centrifuged at 1,500 rpm for 7 min and resuspended in 1 ml of supernatant. Fresh autologous serum (1 ml) was added to each tube, followed by either 7.5 ml of/

of sodium oxamate in one-third strength saline, at a final osmolality of 290 m osmol/kg and concentration of 0.08M, or 7.5 ml of normal saline at an osmolality of 287 m osmol/kg for control purposes. A 0.5 ml volume of Oxford strain staphylococcus in 0.9% NaCl (approximate concentration 50,000 organisms/cu mm) was then added to give a final organism to cell ratio of approximately 1-2:1.

The final 10 ml volumes were then incubated at 37°C for 60 min on an inclined rotatory mixer (33 rpm). They were then centrifuged at 1,500 rpm for 7 min and films spread from the deposit. These were counterstained by May-Grünwald Giemsa and the number of bacteria ingested per neutrophil determined for a total of 300 neutrophils.

3. Results

3.1 EB2 cells Fifteen EB2 cell cultures, with a normal doubling-time of 72 hours, were cultured in the presence of an 0.08M concentration of sodium oxamate in 199 and compared with 10 cultures in the presence of an 0.08M concentration of sodium chloride in 199 at an osmolality of 415-429 m osmol/kg. There were no remaining viable EB2 cells at 48 hours in either system and viable counts were therefore performed at 6 hours and 24 hours (see Table XXVIII). Sodium oxamate, compared with sodium chloride, resulted/

TABLE XXVIII

Percentage of Remaining Viable EB2 Cells when Cultured in the Presence of 0.08M Concentrations of Sodium Oxamate and Sodium Chloride at Two Osmolality Levels. Number of Cultures in Parenthesis.

	0 HR	6 HR	24 HR		0 HR	24 HR	48 HR
Sodium oxamate (15) 415 m osmol/kg	100	$\begin{smallmatrix} +103.4 \\ -46.4 \end{smallmatrix}$	$\begin{smallmatrix} +20.0 \\ -27.3 \end{smallmatrix}$	Sodium oxamate (12) 291 m osmol/kg	100	$\begin{smallmatrix} +65.5 \\ -16.6 \end{smallmatrix}$	$\begin{smallmatrix} +28.4 \\ -14.1 \end{smallmatrix}$
Sodium chloride (10) 429 m osmol/kg	100	$\begin{smallmatrix} +105.5 \\ -9.4 \end{smallmatrix}$	$\begin{smallmatrix} +81.8 \\ -19.1 \end{smallmatrix}$	Sodium chloride (12) 295 m osmol/kg	100	$\begin{smallmatrix} +106.4 \\ -21.0 \end{smallmatrix}$	$\begin{smallmatrix} +76.5 \\ -20.3 \end{smallmatrix}$
Significance of difference of means (P)	-	> 0.5	< 0.01	P	-	< 0.01	< 0.01

resulted in a significant ($P < 0.01$) reduction in viable cells at 24 hours, although not at 6 hours.

Twelve cultures in the presence of 0.08M sodium oxamate were compared with 12 sodium chloride control cultures at the physiological osmolality of 291 and 295 m osmol/kg respectively. An improvement in cell survival was obtained in both systems, but oxamate caused a significant ($P < 0.01$) reduction in viable cells at both 24 and 48 hours compared with the control preparations (see Table XXVIII).

3.2 Marrow lymphoblasts Blast cells from nine patients suffering from acute lymphoblastic leukaemia, newly diagnosed or in haematological relapse, were cultured in the presence of 0.08M sodium oxamate and compared with 11 cultures in the presence of 0.08M sodium chloride, both culture systems giving an osmolality of 415-429 m osmol/kg. Eleven additional cultures were maintained in 199 culture medium but without added oxamate or chloride - the osmolality of this system was 290 m osmol/kg. There was no significant ($P > 0.1$) cytotoxic effect for sodium oxamate compared with sodium chloride at either 24 or 48 hours (see Table XXIX), or for sodium chloride compared with 199 alone ($P > 0.05$).

Eight/

TABLE XXIX

Percentage of Remaining Viable Lymphoblasts when Cultured in 199 and in the Presence of 0.08M Concentrations of Sodium Oxamate and Sodium Chloride at Two Osmolality Levels. Number of Cultures in Parenthesis.

	0 HR	24 HR	48 HR		0 HR	24 HR	48 HR
1. 199 (11) 290 m osmol/kg	100	+72.4 -33.8	+55.1 -37.7	1. 199 (8) 290 m osmol/kg	100	+66.9 -15.8	+63.7 -26.4
2. Sodium chloride (11) 429 m osmol/kg	100	+44.3 -40.9	+26.2 -28.2	2. Sodium chloride (8) 295 m osmol/kg	100	+67.2 -29.7	+57.5 -26.8
3. Sodium oxamate (9) 415 m osmol/kg	100	+24.2 -15.0	+11.2 -13.4	3. Sodium oxamate (8) 291 m osmol/kg	100	+39.7 -15.5	+29.1 -14.0
Significance of difference of 1 v. 2 means (P)		> 0.05 > 0.1	> 0.05 > 0.1	P		> 0.5 < 0.05	> 0.5 < 0.02

Eight additional cultures were established in the presence of sodium oxamate and sodium chloride, at an osmolality of 291-295 m osmol/kg, and in 199 without either additive (see Table XXIX). This resulted in a significant cytotoxic effect for oxamate compared with chloride at both 24 hours ($P < 0.05$) and at 48 hours ($P < 0.02$) but there was no significant ($P > 0.5$) cytotoxic effect for chloride compared with 199 alone.

3.3 Normal marrow myeloid precursors Eleven cultures of normal bone marrow cells were studied in the presence of both sodium oxamate and sodium chloride at 415-429 m osmol/kg, and in 199 without either additive at 290 m osmol/kg. The viable cell counts at 24 and 48 hours were adjusted according to a 500-cell myelogram based on a May-Grünwald Giemsa stained film of the culture. The cell counts for myeloid cells showing cytoplasmic granules (polymorphs, metamyelocytes, myelocytes and promyelocytes) are given in Table XXX. Partially degenerate cells were not included in this myelogram and it was assumed that there was no gross discrepancy in a qualitative sense between viable cells, based on trypan blue exclusion, and Romanowsky-stained cells of normal morphology; the final counts have therefore been presented as viable myeloid cell/

TABLE XXX

Percentage of Remaining Viable Marrow Myeloid Precursors when Cultured in 199 and in the Presence of 0.08M Concentrations of Sodium Oxamate and Sodium Chloride. Number of Cultures in Parenthesis.

	0 HR	24 HR	48 HR		0 HR	24 HR	48 HR
1. 199 (11) 290 m osmol/kg	100	84.0 +18.0	72.9 +19.8	1. 199 (10) 290 m osmol/kg	100	89.7 +27.5	84.8 +28.2
2. Sodium chloride (11) 429 m osmol/kg	100	59.6 +22.9	47.6 +16.5	2. Sodium chloride (10) 295 m osmol/kg	100	86.9 +23.2	85.9 +25.9
3. Sodium oxamate (11) 415 m osmol/kg	100	79.6 +16.2	60.2 +17.2	3. Sodium oxamate (10) 291 m osmol/kg	100	89.1 +26.1	83.5 +23.6
Significance of difference of 1 v. 2 means (P)		<0.02 <0.05	<0.01 >0.05	P		>0.5 >0.5	>0.5 >0.5

cell counts (see Table XXX). The sodium chloride culture showed a significant reduction, compared with 199 alone, in viable myeloid cells at 24 hours ($P < 0.02$) and 48 hours ($P < 0.01$), and also showed a reduction in viable cells compared with the sodium oxamate culture at 24 hours ($P < 0.05$), but not at 48 hours ($P > 0.05$). This is presumed to be an osmotic effect due to the slightly higher osmolality of the sodium chloride culture system.

Ten cultures of each system at physiological osmolality showed improved cell survival but with no significant ($P > 0.5$) difference between sodium oxamate and sodium chloride or between sodium chloride and 199 alone at either 24 or 48 hours (see Table XXX).

3.4 Blood lymphocytes Ten cultures of normal blood lymphocytes showed a significant ($P < 0.05$) reduction in viable cells in sodium chloride compared with 199, and in sodium chloride compared with sodium oxamate, at high osmolality at both 24 and 48 hours (see Table XXXI). This was again considered to be an osmotic effect.

When 10 additional cultures were studied at physiological osmolality there was no significant difference between sodium oxamate and sodium chloride ($P > 0.1$) or between sodium chloride and 199 alone ($P > 0.05$) - see/

TABLE XXXI

Percentage of Remaining Viable Blood Lymphocytes when Cultured in 199 and in the Presence of 0.08M Concentrations of Sodium Oxamate and Sodium Chloride at Two Osmolality Levels. Number of Cultures in Parenthesis

	0 HR	24 HR	48 HR		0 HR	24 HR	48 HR
1. 199 (11) 290 m osmol/kg	100	$\begin{smallmatrix} +87.3 \\ -17.9 \end{smallmatrix}$	$\begin{smallmatrix} +76.8 \\ -18.9 \end{smallmatrix}$	1. 199 (10) 290 m osmol/kg	100	$\begin{smallmatrix} +82.7 \\ -11.1 \end{smallmatrix}$	$\begin{smallmatrix} +87.1 \\ -6.9 \end{smallmatrix}$
2. Sodium chloride (10) 429 m osmol/kg	100	$\begin{smallmatrix} +55.0 \\ -35.2 \end{smallmatrix}$	$\begin{smallmatrix} +23.6 \\ -21.0 \end{smallmatrix}$	2. Sodium chloride (10) 295 m osmol/kg	100	$\begin{smallmatrix} +91.2 \\ -16.4 \end{smallmatrix}$	$\begin{smallmatrix} +80.9 \\ -8.7 \end{smallmatrix}$
3. Sodium oxamate (11) 415 m osmol/kg	100	$\begin{smallmatrix} +83.2 \\ -22.9 \end{smallmatrix}$	$\begin{smallmatrix} +56.8 \\ -26.4 \end{smallmatrix}$	3. Sodium oxamate (10) 291 m osmol/kg	100	$\begin{smallmatrix} +101.1 \\ -17.2 \end{smallmatrix}$	$\begin{smallmatrix} +89.6 \\ -14.6 \end{smallmatrix}$
Significance of difference of means (p)		$\begin{smallmatrix} <0.02 \\ <0.05 \end{smallmatrix}$	$\begin{smallmatrix} <0.01 \\ <0.01 \end{smallmatrix}$	P		$\begin{smallmatrix} >0.1 \\ >0.1 \end{smallmatrix}$	$\begin{smallmatrix} >0.05 \\ >0.1 \end{smallmatrix}$

TABLE XXXII

Percentage of Remaining Viable Blood Neutrophils when Cultured in 199 and in the Presence of 0.08M Concentrations of Sodium Oxamate and Sodium Chloride at Two Osmolality Levels. Number of Cultures in Parenthesis

	0 HR	24 HR		0 HR	24 HR	48 HR
1. 199 (11) 290 m osmol/kg	100	+73.7 -23.2	1. 199 (10) 290 m osmol/kg	100	+83.3 -12.3	+62.1 -15.8
2. Sodium chloride (11) 429 m osmol/kg	100	+10.3 -7.2	2. Sodium chloride (10) 295 m osmol/kg	100	+80.1 -12.1	+63.5 -11.9
3. Sodium oxamate (12) 415 m osmol/kg	100	+18.0 -13.3	3. Sodium oxamate (10) 291 m osmol/kg	100	+84.3 -13.1	+56.7 -18.8
Significance of difference of means (P)		<0.01 >0.05	P		>0.5 >0.1	>0.5 >0.1

see Table XXXI.

3.5 Blood neutrophils Twelve cultures of blood neutrophils in the presence of 0.08M sodium oxamate and 11 cultures in the presence of 0.08M sodium chloride were compared at an osmolality of 415-429 m osmol/kg (see Table XXXII). There was a considerable and significant ($P < 0.01$) reduction in viable cell count in both systems by 24 hours, but with no significant ($P > 0.05$) difference between sodium oxamate and sodium chloride. There were virtually no remaining viable cells at 48 hours.

Ten cultures in the presence of sodium oxamate and 10 in the presence of sodium chloride were compared at an osmolality of 291-295 m osmol/kg (see Table XXXII). There was a considerable improvement in cell viability but with no significant difference ($P > 0.1$) between the two systems at 24 and 48 hours.

3.6 Neutrophil phagocytosis The number of staphylococci ingested by neutrophils during culture for 60 min in a medium containing 0.08M sodium oxamate was 1.00 ± 0.32 organisms per cell (mean \pm SD for 10 estimations) compared with 1.25 ± 0.45 organisms per cell when cultured in the presence of sodium chloride at equivalent osmolality. The mean figure for bacterial ingestion was significantly ($P < 0.02$)/

($P < 0.02$) lower in the presence of oxamate. The mean percentage of neutrophils containing phagocytosed bacteria was not significantly ($P > 0.5$) different between the two systems ($56.4 \pm 9.1\%$ and $59.2 \pm 5.0\%$ respectively).

4. Discussion

The concentration of sodium oxamate (0.08M) used in these experiments was derived from the concentration (0.04-0.08M) reported to inhibit the LD activity of tumour cell extracts and inhibit the glycolytic activity of intact Ehrlich ascites tumour cells (Papaconstantinou and Colowick, 1961a). This concentration was also found to inhibit the growth of Hela cells (Papaconstantinou and Colowick, 1961b). The possible cytotoxic effect of lower concentrations on leukaemic cells was not investigated in these experiments.

Sodium iodoacetate, at a concentration of 0.001-0.01M, is a more potent inhibitor of aerobic lactic acid production compared with oxamate at a concentration of 0.01-0.1M (McKinney and Martin, 1956). Iodoacetate acts, however, by inhibiting the action of glyceraldehyde-phosphate (triosephosphate) dehydrogenase (see Fig.2) and this also interferes with the oxidation of carbohydrate via pyruvic acid and the tricarboxylic acid cycle. Thus oxamate/

oxamate has the advantage of a selective inhibition of glycolysis and does not interfere with mitochondrial oxidation (Papaconstantinou and Colowick, 1961a).

The relatively high glycolytic enzyme activity of the lymphoblast together with its low intra-mitochondrial enzyme activity suggested that these cells might be abnormally sensitive to the cytotoxic effect of a glycolytic enzyme inhibitor. Although the lymphoblast can demonstrate an oxidative metabolism (Seitz and Luganova, 1968) the small number of mitochondria must establish an upper limit to its respiratory rate. It was hoped that under conditions of glycolytic enzyme inhibition these cells would retain sufficient oxidative metabolism to prevent pyruvate accumulation and competitive inhibition of the oxamate effect, but would not produce sufficient ATP by this route to substitute for glycolysis.

The relatively high osmolarity of oxamate in these experiments made it difficult to differentiate its potential cytotoxic effect, due to LD inhibition, from the osmotic disruptive effect. Although an attempt was made to assess this differential effect by adding an equimolar concentration of sodium chloride to control cultures, the effect of adding either sodium oxamate or sodium chloride at a concentration of 0.08M to 199 culture medium/

medium is to produce an unphysiological osmolality of 415-429 m osmol/kg. This resulted in a relatively high cell death rate within 24-48 hours. Under these conditions the cytotoxic effect of oxamate, compared with sodium chloride, was significant only for EB2 cells at 24 hours.

When the 199 culture medium was diluted to one-third strength with distilled water then the final osmolality in the presence of added sodium oxamate and sodium chloride fell into the physiological range at 291-295 m osmol/kg and a general improvement in cell viability resulted. At this osmolality, sodium oxamate showed a significant cytotoxic effect, relative to sodium chloride, for both EB2 cells and leukaemic lymphoblasts at 24 and 48 hours. No significant oxamate cytotoxic effect was obtained for normal marrow myeloid cell precursors or for blood lymphocytes and neutrophils. This indicates that these normal cells are either less dependent on glycolysis for ATP synthesis and/or possess a greater potential for oxidative phosphorylation due, perhaps, in the case of myeloid precursors, to the possession of a larger number of mitochondria.

The normal polymorph would appear to have the enzyme apparatus/

apparatus to follow either a glycolytic or a respiratory type of metabolism in vivo, although the former probably predominates. A higher level of aerobic glycolysis results when there is an adverse environmental change and during particle ingestion (Selvarajp and Sbarra, 1966). The extent to which aerobic glycolysis does predominate in vivo is in doubt and the controversy hinges on the extent to which the very high glycolytic activity demonstrated in vitro by some workers reflects the effect of cell injury during the separation procedure (Beck, 1968). It is probable that both glycolysis and oxidative phosphorylation are available for ATP synthesis and that either pathway may compensate for decreased activity of the other for purposes of cell survival. For this reason oxamate does not have a significant cytotoxic effect. Particle ingestion, however, is apparently solely dependent on glycolysis and this may explain the effect of oxamate in suppressing staphylococcal ingestion. Sbarra and Karnovsky (1959) also found that the glycolytic inhibitor iodoacetate decreased polymorph phagocytosis in parallel with depression of glycolysis. The clinical significance of this reduction in phagocytosis in the presence of an 0.08M concentration of oxamate remains to be determined.

It/

It would appear, from the results of these experiments, that LD inhibition may result in a fairly selective cytotoxic effect for those haemopoietic cells which show a high glycolytic enzyme activity and also contain scanty mitochondria with resulting weak respiratory enzyme activity. The results of the enzyme studies suggest that only the lymphoblast and the Burkitt lymphoma cell will be susceptible to LD inhibition although the chronic lymphocytic leukaemia lymphocyte may be partially sensitive. The myeloblast, in general, contains a larger number of mitochondria than the lymphoblast (Freeman and Journey, 1971) and, in these studies, showed a higher respiratory enzyme activity. The relatively undifferentiated myeloblast, however, appears to contain fewer mitochondria than the more differentiated myeloblast, which is closer to the promyelocyte in ultrastructural appearance (Kakefuda, 1968; Freeman, 1971). Freeman and Journey (1971) also observed a similar variation in mitochondrial number within the morphological range of myelomonoblasts with a greater number of mitochondria in more differentiated cells; the more undifferentiated, or stem-cell, types of leukaemia may therefore also be sensitive to LD inhibition.

**MAIN CONCLUSIONS AND POTENTIAL VALUE
OF THE OBSERVATIONS**

The main value of enzyme cytochemical studies in haemopoietic cells is the ability to relate enzyme activity to the stage of cell maturation. Solitary estimations of individual enzymes are of limited biological meaning, whether expressed in arbitrary microdensitometer or other units, and the experiments described in this thesis were therefore designed to include representative enzymes from several pathways of carbohydrate metabolism and to study their inter-relationships in parallel with cell maturation. Since one of these pathways is physically enclosed behind a mitochondrial membrane of selective permeability this involved a study of mitochondrial membrane hydrogen transport systems.

The results suggest that in human haemopoietic cells the demonstrable intra-mitochondrial and α -glycerol-phosphate shuttle system enzyme activity is proportional to the number of mitochondria within the cell.

Immature lymphoid cells have low intra-mitochondrial and α -glycerolphosphate shuttle enzyme activity, contain scanty mitochondria and are probably dependent on extra-mitochondrial glycolysis, and in particular LD activity, for NADH_2 reoxidation and ATP synthesis. Since the lymphoblast also shows low pentose phosphate shunt enzyme activity/

activity, relative to the higher potential indicated by NADPH_2 -diaphorase activity, together with increased transhydrogenase activity indicating redeployment of hydrogen from pentose phosphate-generated NADPH_2 to form NADH_2 , this additional source of NADH_2 further increases the metabolic dependence on LD. This implies that selective LD inhibition might have a preferential cytotoxic effect for the lymphoblast, and also the EB2 cell, rather than for more mature marrow cells.

Although the data obtained from the in vitro cytotoxicity accords closely with the predicted oxamate sensitivity, based on the enzyme studies, it cannot be assumed that the cytotoxic action of oxamate was solely due to its known role as a specific LD inhibitor; this requires further investigation.

Since the lymphoblast shows disproportionately high glycolytic enzyme (LD) activity and since mitosis is dependent on glycolysis, then LD inhibition may have a potential in vivo cytotoxic effect. Goldberg and Colowick (1965) have already provided evidence to show that the effect of oxamate on the growth of Hela cells is related to the stage of mitotic cycle. There is evidence that other cytotoxic agents may also interfere with glycolysis-dependent mitosis. Hunter (1963) suggested/

suggested that vinca alkaloids produce metaphase arrest by inhibition of the Pasteur effect and therefore the energy production necessary for mitosis. Hydrocortisone in high concentration is an effective NADH_2 -oxidase inhibitor (Mandell, Rubin and Hook, 1970) and since the end-result of this is similar to a failure to reoxidise NADH_2 via the mitochondrial respiratory pathway, the effect of corticosteroids may enhance oxamate cytotoxicity by increasing metabolic dependence on LD for NADH_2 reoxidation. Therefore any in vivo cytotoxic action of oxamate should be studied in combination with corticosteroids and vinca alkaloids in anticipation of a potential synergistic effect.

The potential in vivo effect of oxamate in acute lymphoblastic leukaemia will depend on the extent to which the blast cell is dependent on mitochondrial respiratory ATP synthesis for survival when it requires to exist as an obligatory aerobe due to the action of oxamate. Despite the low activity of the α -glycerolphosphate shuttle enzymes there is an appreciable activity in lymphoblasts of the other, possibly alternative, shuttle systems catalysed by β -HED and MD. Although these alternative shuttle systems may be able to compensate for the low activity of α -GPD-NAD, the number of mitochondria/

mitochondria and therefore aerobic ATP synthesis, is more likely to be the final determining factor of sensitivity to oxamate. If, like the small lymphocyte in response to plant mitogens, the lymphoblast is capable of forming additional mitochondria in response to an increased demand for respiration then the in vivo cytotoxicity of oxamate is likely to be limited.

In order to achieve satisfactory in vivo levels of oxamate it may be necessary to develop an oxamate analogue which is not excreted as rapidly as oxamate itself (Papaconstantinou and Colowick, 1961b). Oxamate also has the potential disadvantage that it may result in an anticoagulant effect by virtue of conversion to oxalic acid. Alternative methods of LD inhibition - for example, the production of an antibody against LD (Ng and Gregory, 1968) or the use of a specific natural peptide with inhibitory properties (Walker and Schoenenberger, 1966) - may therefore have a greater potential clinical value.

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- January to July, 1965 : Visiting Research Fellow,
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- January, 1966 to July, 1967 : Lecturer in Haematology,
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- January, 1969 to July, 1971 : Consultant Haematologist,
The Children's Hospital,
Birmingham, 16 and Honorary
Lecturer, Department of
Experimental Pathology,
University of Birmingham.

My/

My own contribution to this thesis includes: the original idea to study dehydrogenase enzymes and mitochondrial membrane shuttle systems in normal and leukaemic cells; the design of all subsequent experiments; the development of the cytochemical techniques (including the technical work); the microscopic examination and visual scoring of all marrow and blood films; the quantitative measurements, by microdensitometry, of formazan production; the compilation of the tables, graphs and photographs; and the composition of the text.

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A handwritten signature in dark ink, appearing to be 'John A. L.', written in a cursive style with a long horizontal stroke extending to the right.

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STATISTICAL METHODS

Statistical significance values were determined by means of Bessel's standard deviation and Student's 't' test using the following formulae:

$$\text{Standard deviation } (\sigma) = \sqrt{\frac{n (\sum x^2) - (\sum \bar{x})^2}{n^2}}$$

$$\text{Bessel's corrected standard deviation} = \sqrt{\sigma^2 \times \frac{n}{n-1}}$$

The majority of calculations were performed using a programmed Friden electronic calculator. Where 'significance' values are quoted as, for example, $P < 0.05$ this represents $0.05 > P > 0.02$; for greater degrees of significance the P value is expressly stated as < 0.02 or < 0.01 .

SOURCE LIST OF CHEMICALS AND EQUIPMENT

Co-enzymes : Boehringer

NAD : nicotinamide adenine dinucleotide, free acid

NADH₂ : reduced nicotinamide adenine dinucleotide, disodium salt

NADP : nicotinamide adenine dinucleotide phosphate, disodium salt

NADPH₂ : reduced nicotinamide adenine dinucleotide, tetrasodium salt

Miscellaneous Chemicals

Collagen polypeptide: British Glues and Chemicals Ltd., London, S.E.1.

Ficoll: Pharmacia (G.B.) Ltd., 75 Uxbridge Road, London, W.5.

Glycylglycine: Hopkin and Williams, Essex.

MTT: Sigma Chemical Co. Ltd., London.

NBT: Sigma Chemical Co. Ltd., London.

PVA: British Drug Houses, Poole, and Bush, Beach and Segner Bayley Ltd., 175 Tottenham Court Road, London, W1P 0BJ.

Sodium oxamate: Newhaven Technical Supply Company Ltd., Beach Road, Newhaven, Sussex.

Substrates for specific enzymes

LD: Sodium lactate solution; 72% w/w (British Drug Houses)

α -GPD and α -GPD-NAD: DL- α -glycerolphosphate, disodium salt (Sigma)

β /

β -HBD:	DL- β -hydroxybutyric acid, sodium salt (Sigma)
GD:	L-glutamic acid, sodium salt (Koch Light)
MD:	sodium malate (Hopkin and Williams)
SD:	sodium succinate (Hopkin and Williams)
G6PD:	D-glucose-6-phosphate, disodium salt (Boehringer)
6PGD:	6-phosphogluconic acid, trisodium salt (Boehringer)

Equipment

Hyperbaric tank:	Drury Engineering Co. Ltd., Walsall.
Incubator-humidity box: and perspex rings for 'open-ring' technique	Barton Scientific Co., Barton-le-Clay, Bedfordshire.
Osmometer:	Advanced Instruments Inc., 45 Kenneth Street, Newton Highlands, Massachusetts, 02161, U.S.A.
Scanning and integrating microdensitometer:	Barr and Stroud Ltd., Caxton Street, Anniesland, Glasgow, W.3.